# MOLECULAR AND CELL GENETIC ANALYSIS OF CHROMOSOME 11 IN SPORADIC EPITHELIAL OVARIAN CANCER

Hani Gabra

**Ph.D.**

**University of Edinburgh**

**ICRF Medical Oncology Unit**

**and**

**MRC Human Genetics Unit**

**Edinburgh**

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I declare that this thesis has been composed entirely by myself and that this is my own work except where I have indicated the contribution of others

Hani Gabra
October 1996

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## Abstract

Ovarian cancer causes over 4,200 deaths in the U.K. per annum. This is because it presents late, resulting in an extremely poor prognosis. Tumour-suppressor genes(TSGs) are intimately involved in carcinogenesis. In sporadic ovarian cancer, few important genes of this type have been described.

To investigate new TSG regions, I used PCR based polymorphic microsatellites to define regions of frequent loss of heterozygosity (LOH) throughout chromosome 11 especially at 11p15.5-15.1 and 11q23-qter in DNA blood/tumour pairs from 60 patients with ovarian neoplasms. LOH at 11q23-qter was significantly associated with advanced stage and adverse actuarial survival. Integration of this LOH data with chromosome 17 LOH data allowed construction of a multistep model for ovarian cancer. Refinement of the 11q23-qter region revealed an 8.5Mb LOH region between *D11S934* and *D11S1320* (11q23.3-q24.3) retaining the above survival association.

To test the causality of the LOH observations, I introduced normal chr 11 by microcell mediated chromosome transfer (MMCT) into an ovarian cancer cell line. I used 556.1.5, a human monochromosome somatic cell hybrid to transfer neor-tagged chr 11 into a Hygr subline of OVCAR3 (OH3), an ovarian cancer line with a chr 11 rearrangement. I have analysed 14 microcell hybrid clones (MHCs) from 5 experiments. Chr 11 transfer was confirmed by PCR, *in-situ* hybridisation and microsatellite analysis.
Interestingly, chr 12 MMCT, originally intended as a negative control was shown to inhibit the growth of OH3 *in-vitro* and *in-vivo* suggesting the presence of a TSG on this chromosome also. Appropriate controls were generated by transfer of a "neutral" chromosome from neo-transfected OVCAR3 by MMCT to OH3.

Chr11 MHCs remained immortalised, and demonstrated significant *in-vitro* growth inhibition. This inhibition was not due to cell cycle block (DNA FACS analysis) or apoptosis (FITC labelled

Annexin-V and fluoro-TUNEL FACS analysis). MHCs were significantly reduced in their ability to invade matrigel in the transwell invasion assay. MHCs retaining whole chr 11 showed significant reduction in invasiveness compared with MHCs taking up all chr 11 as far as 11q22. Soft agar clonogenicity was unaltered. Xenografts of chr 11 MHCs showed reduced growth rather than suppression of tumorigenicity.

Further analysis of the inhibition of invasiveness associated with the introduction of 11q revealed that components of this include specific inhibition of attachment and spreading to laminin coated plastic, and abrogation of the stimulation of tumour cell migration in response to a collagen IV or fibronectin mediated haptotactic signal.

These studies suggest that the gene within a 4.5 Mb interval on distal chromosome 11q24 functions as an ovarian cancer progression-suppressor. This analysis provides a starting point for an integrated positional/functional cloning approach.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ALF | Automated laser fluorescence |
| Amp | Ampicillin |
| ANOVA | Analysis of variance |
| AT | Ataxia telangiectasia |
| bp | base pairs |
| BSA | Bovine serum albumin |
| C | Centigrade |
| C IV | Collagen IV |
| CA-125 | ovarian cancer antigen CA-125 |
| CCD | Charge coupled device |
| CDDP | Cis-di-amino-dichloro-platinum |
| CDKI | Cyclin dependent kinase inhibitor |
| cDNA | Complementary deoxyribonucleic acid |
| CGH | Comparative genomic hybridisation |
| CHO | Chinese hamster ovary |
| Ci | Curies |
| cM | centiMorgan |
| CMGT | Chromosome-mediated gene transfer |
| CP | Cyclophosphamide and Platinum |
| DAPI | 4,6-diamidino-2-phenylindole |
| dATP | deoxyadenosine triphosphate |
| DCC | Deleted in colorectal cancer |
| dCTP | deoxycytidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| DMEM | Dulbecco's modified minimal essential medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| ds DNA | Double stranded DNA |
| dTTP | deoxythymidine triphosphate |
| dUTP | deoxyuridine triphosphate |
| ECM | Extra-cellular matrix |
| EDTA | Ethylene di-amine tetra-acetic acid |
| EHS | Engelbreth-Holm-Swarm |
| ELISA | Enzyme linked immuno-sorbent-assay |
| EOC | Epithelial ovarian cancer |
| ERCC | Excision repair cross-complementing |
| FACS | fluorescence activated cell sorter |
| FCS | Fetal calf serum |
| FGF | fibroblast growth factor |
| FIGO | International Federation of Obstetrics and Gynaecology |
| FISH | Fluorescence in-situ hybridisation |
| FITC | Fluorescein isothiocyanate |

| | |
|---|---|
| FN | Fibronectin |
| g | Grams |
| G418 | Geneticin |
| GOG | Gynecologic Oncology Group |
| HET | Heterozygous, no-loss |
| hmB | hygromycin B |
| HPRT | Hypoxanthine-guanine phosphoribosyl transferase |
| hr | hours |
| ICRF | Imperial Cancer Research Fund |
| IFGT | Irradiation-fusion gene-transfer |
| IGF2 | insulin-like growth factor |
| INS | Insulin |
| IRS-PCR | Interspersed-repetitive-sequence PCR |
| J1Cl4 | J1 clone 4 |
| Kb | kilobases |
| kD | kilodalton |
| L-Broth | Luria Broth |
| L1 | Line 1 |
| LacO | Lac operator |
| Leu2 | Leucine |
| LINE | Long interspersed nuclear element |
| LMP | Low malgnant potential |
| LN | Laminin |
| LOH | Loss of heterozygosity |
| LR | Laminin receptor |
| M | Molar |
| Mb | Megabases |
| MHC | Microcell hybrid clone |
| min | minutes |
| ml | millilitres |
| MMCT | Microcell-mediated chromosome transfer |
| MMP | Matrix metallo-proteinase |
| MRC HGU | Medical Research Council Human Genetics Unit |
| mRNA | messenger RNA |
| MSI | Microsatellite instability |
| MSP | Microsatellite polymorphism |
| MTT | 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide |
| NCAM | Neural cell adhesion molecule |
| neo | Neomycin |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCNA | proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |

| | |
|---|---|
| PFGE | Pulse-field gel-electrophoresis |
| PGR | Progesterone receptor |
| PHAP | Phytohaemagglutinin-P |
| PI | Propidium iodide |
| PNS | Positive-negative selection |
| PRINS | Primed in-situ hybridisation |
| PVP | Polyvinyl pyrrolidine |
| RB1 | Retinoblastoma gene |
| RDA | Representational difference analysis |
| RFLP | Restriction fragment length polymorphism |
| RH | radiation hybrid |
| RNA | ribonucleic acid |
| RNAse | Ribonuclease |
| rpm | Revolutions per minute |
| RT-PCR | Reverse-transcription PCR |
| s, sec | seconds |
| SCID | Severe combined immunodeficiency |
| SDS | Sodium dodecyl sulphate |
| SINE | Short interspersed nuclear element |
| SRO | Shortest region of overlap |
| SSCP | Single stranded conformational polymorphism |
| STS | sequence tagged site |
| TdT | Terminal deoxynucleotide transferase |
| TGF | Transforming growth factor |
| TK | Thymidine kinase |
| TP | Taxol and Platinum |
| tRNA | transfer RNA |
| TSG | Tumour suppressor gene |
| UV | Ultra violet |
| WT | Wilms' tumour |
| XMMCT | Irradiation microcell-mediated chromosome transfer |
| YAC | Yeast artificial chromosome |

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# 1. INTRODUCTION

## 1.1 Epithelial ovarian cancer: causation and disease distribution

### 1.1.1 Epidemiology

Ovarian cancer is the most common cause of death from gynaecological malignancy, and the fifth commonest cause of cancer death in women after breast, lung, colon and (in the UK) stomach cancer. About 5,000 cases occur annually in the UK, with approximately 4,200 deaths in the same period (Chang et al, 1994). This high mortality rate is due to presentation at an advanced stage of the disease as symptoms of the disease are insidious in onset and non-specific in nature. Approximately 1 woman in 70 will develop ovarian cancer, and 1 woman in 100 will die from it. Incidence is age-related, peaking at 55 per 100,000 per year in the 7th and 8th decades, with the disease being uncommon in those under 45 (less than 15 cases per 100,000 per year). The overall five-year survival rate is poor; about 30%, primarily due to the late presenting nature of the disease. There is a particularly high incidence in Scandinavia, followed by Northern Europe and North America. Despite being industrialised, Japan has a particularly low incidence of the disease; however, the incidence increases sharply in Japanese migrants to the USA and their daughters (a phenomenon seen in common with other cancers), suggesting profound environmental components to aetiology (Herrinton et al, 1994).

Demographically, there are wide mortality rate differences; Denmark having a mortality rate six times that of Japan. These may be due to genetic differences between populations (Parazzini et al, 1991)

There has been a slight improvement in ovarian cancer survival over the last two decades, almost certainly due to improved multimodality therapies. This improvement has been more pronounced in younger patients (less than 50 years old). The age-specific mortality rate curve in those with ovarian cancer becomes parallel to that of the general age-specific mortality rate at 15-20 years post diagnosis, indicating those who may be "cured" of their disease by that time.

### 1.1.2 Aetiology

Ninety-five percent of all patients with ovarian cancer have no family history, suggesting that almost all cases can be attributed to spontaneous or environmentally induced carcinogenesis. Many individual environmental factors have been examined. The epidemiological studies that are published for these potential factors are diverse in their methodologies and power. What is clear is that for many factors, the epidemiological data is (a) conflicting from study to study and (b) small in magnitude of effect, often at the limits of what is legitimately detectable by the study designs. Putting these points together with that of publication bias gives an idea of how opaque this area continues to be. Potential candidate carcinogens such as tobacco (Whittemore et al, 1988 ), ascending (talc) (Cramer et al, 1982a; Harlow et al, 1992; Wehner et al, 1994) asbestos, alcohol (Gwinn et al, 1986; Kato et al, 1989) have been examined and do not seem to confer excess risk.

The low incidence of the disease in Japan does suggest that the causes are not simply due to environmental carcinogens as a result of industrialisation; however, the rising incidence in Japanese migrants to the USA suggests environmentally related carcinogenesis. Whether this is due to carcinogenesis from, say, dietary or other sources in the USA, or whether there are dietary or other protectants that prevent environmentally associated carcinogenesis in Japan is unknown.

Coffee drinking has been variously ascribed with slightly increased risk or no risk depending on the epidemiological overview or opinion, and there may be some consensus that a slightly increased risk of ovarian cancer is associated with coffee ingestion (Byers et al, 1983; Cramer et al, 1984; Whittemore et al, 1988).

Initial evidence that consumption of animal fat is associated with an increased risk of ovarian cancer (Cramer et al, 1984), was followed with a large case/control study into diet and ovarian cancer risk. This study suggested that every 10g of ingested saturated fat per day increased ovarian cancer risk by 20%, whereas the same ingested weight of vegetable fibre reduced ovarian cancer risk by 37% (Risch et al, 1994).

The extent of independence of these factors is unclear. However, this seemingly unambiguous study is unique, there are very few other confirmatory studies, and meta-analytically its findings should be treated cautiously.

Although no relationship has been shown between the exogenous ingestion of oestrogen and the incidence of ovarian cancer (Kaufman et al, 1989), re-examination of this area may be warranted with these new observations because of the role of oestrogens and other steroid hormones often present in saturated animal fat (Risch et al, 1994), the role of phytoestrogens acting to inhibit endogenous oestrogen production, (Adlercreutz et al, 1992; Adlercreutz et al, 1993; Mousavi et al, 1993) and the role of vegetable fibre in binding oestrogen in the gut (Rose et al, 1991; Goldin et al, 1994).

During the late 1980's, a case/control study was performed which showed an association between higher consumption of Lactose/Galactose (in the form of yoghurt and cottage cheese) and lower levels of the enzyme galactose-1-phosphate uridyl transferase in erythrocytes in ovarian cancer cases compared with controls (Cramer et al, 1989). Deficiency of this enzyme may be a genetic risk factor for early menopause. Early menopause may be associated with hypergonadotrophic hypogonadism, and such hormonal perturbation may be involved in the patho-aetiology of ovarian cancer (Cramer et al, 1990). This work awaits confirmation by follow-up studies.

### 1.1.3 Hormonal factors and incessant ovulation

Although no formal evidence exists to suggest that exogenous oestrogens or other steroid hormones have a role to play in the initiation of ovarian cancer in humans, there is quite a body of literature that would suggest that ovarian cancer is hormone sensitive. The ovary is the main site of synthesis of oestrogen and progesterone and is also a target organ for these hormones. The actions of these hormones are mediated by specific intracellular receptors which function as hormone-inducible nuclear transcription factors with

context-specific, often conflicting effects on proliferation and differentiation of target tissues (O'Malley et al, 1990).

Progesterone receptor (PR), whose gene is located on chromosome 11q22-q23 (Rousseau-Merck et al, 1987), is regulated by oestradiol via the oestrogen receptor (ER) (Horwitz et al, 1978). Oestradiol induced PR expression has been demonstrated in ovarian cancer cell lines which express ER (Langdon et al, 1994).

Ovarian cancer has been reported to respond to antioestrogens in about 10-20% of cases, and to progestins with an average of 36%, within a range between 0% and 60% (Slotman et al, 1988; Bonte et al, 1979).

That histopathological subtypes of epithelial ovarian cancer (EOC) differ in their tumoural PR expression is suggested by at least 6 reports that endometrioid ovarian carcinomas contain relatively more PR than other histological types, (Slotman et al, 1988) and that PR positivity is associated with well differentiated ovarian tumours in premenopausal women (Friedman et al, 1979). Tumoural PR content has prognostic significance in several studies, (Sevelda et al, 1990; Slotman et al, 1988) but the associations are not unequivocal (Rose et al, 1990; Masood et al, 1989).

Oestrogen receptors have been measured in ovarian cancer in many studies; the evidence for an association of tumoral ER content with prognosis is conflicting (Kieback et al, 1993a; Kieback et al, 1993b; Masood et al, 1989; Sevelda et al, 1990 ).

Although exogenous oestrogens do not appear to be associated with the development of human ovarian carcinoma (despite evidence that chronic oestrogen and progestin administration in animal studies results in ovarian cancer (Gardner et al, 1958; Jabara et al, 1962), hormonal perturbations with consequent effects on ovulation appear to profoundly affect the development of ovarian cancer, as predicted by the hypothesis of "Incessant ovulation". To this extent, the aetiology of ovarian cancer may furthermore be considered to be endocrine related.

In 1971, Fathalla proposed that "Incessant ovulation" results in repeated proliferative repair cycles of damaged ovarian surface epithelium (Fathalla, 1971). These epithelial cells can acquire genetic damage during proliferative repair, and this cumulative genetic damage due to repeated ovulatory cycles predisposes to the development of ovarian cancer. This hypothesis is perfectly understandable if one considers that in a panmyctic natural state a female might only ovulate 20-40 times in her life due to recurrent childbearing, whereas in late twentieth-century economically-developed societies, this figure may be an order of magnitude higher.

Thus any factor which reduces the number of times a woman ovulates (late menarche, oral contraceptive use, multigravidity, prolonged lactation, early menopause) can be predicted to reduce ovarian cancer risk.

Nulliparity and a low mean number of pregnancies have been associated with increased risk (Cramer et al, 1983). Conversely, childbirth confers a protective effect, and the magnitude of this protective effect relates to parity with an estimated 30-60% reduction in risk for those women with 2 or more pregnancies.

Increased total pregnancy and lactation time also confer a protective effect (Weiss et al, 1981).

Ovarian cancer risk is decreased 30-60% by the use of oral contraceptives; in one study, ingestion of oral contraceptive over a 5 year period was associated with 37% reduction in ovarian cancer risk; this effect was observed to persist for several years after cessation of the oral contraceptive (Cramer et al, 1982b). Therefore the hypothesis is consistent with the idea that by reducing the number of ovulations and their associated damage-repair cycles the risk of ovarian cancer is lowered.

The high gonadotrophic milieu of the post-menopausal state may confer additional risk (Cramer et al, 1983)

Fertility drugs (exogenous gonadotrophins and stimulants of pituitary gonadotrophins which act by ovarian hyperstimulation) have been suggested to confer increased risk (as might be expected from the Fathalla hypothesis); however the evidence is not unequivocal. A small cohort study showed no apparent increase in risk of ovarian cancer, although there was an increase in endometrial cancer (Ron et al, 1987). A subsequent review of 12 American case/control studies suggested a threefold increase risk of ovarian cancer in women who had used fertility drugs (Whittemore et al, 1992; Whittemore et al, 1993). However, it has been pointed out that this was a small study with wide confidence intervals; in addition, controls lacked a history of infertility, and this presents a problem as nulliparous women are intrinsically at increased ovarian cancer risk (Spirtas et al, 1993). Recently, another case/control study in nearly 4000 women reported that women treated with infertility drugs had a 2.5 times increased risk of ovarian cancer compared to the general population (Rossing et al, 1994). Women undergoing fertility treatment therefore need to know that they may be at increased risk of ovarian cancer, although this information requires careful presentation management given its equivocacy.

## 1.2 Epithelial ovarian cancer: biology and pathophysiology

### 1.2.1 Developmental histogenesis

The bewildering array of different neoplasms arising from the ovary can be understood if one considers the embryological development of the female reproductive system, the range of epithelial tissue types that it generates, and the physiology of the ovary (Langman, 1975).

As early as the fourth week after fertilisation, the developing gonads commence as the genital ridge in the embryo by a proliferation of coelomic epithelium (a cell layer of mesodermal origin which goes on to form the peritoneal epithelial lining). Primordial germ cells appear at week six and migrate from the wall of the yolk sac to the genital ridge.

As the germ cells invade the genital ridge, the overlying coelomic epithelium proliferates and invades the underlying mesenchyme where it forms intimate relationships, surrounding the germ cells in structures called sex-cords. As the ovary develops the sex-cords form irregular clusters and the central part of the ovary loses the cords. These are replaced by vascular stroma and become the ovarian medulla. The cords proliferate in the cortical region and form oogonia while the surrounding epithelial cells form the enveloping follicular cells.

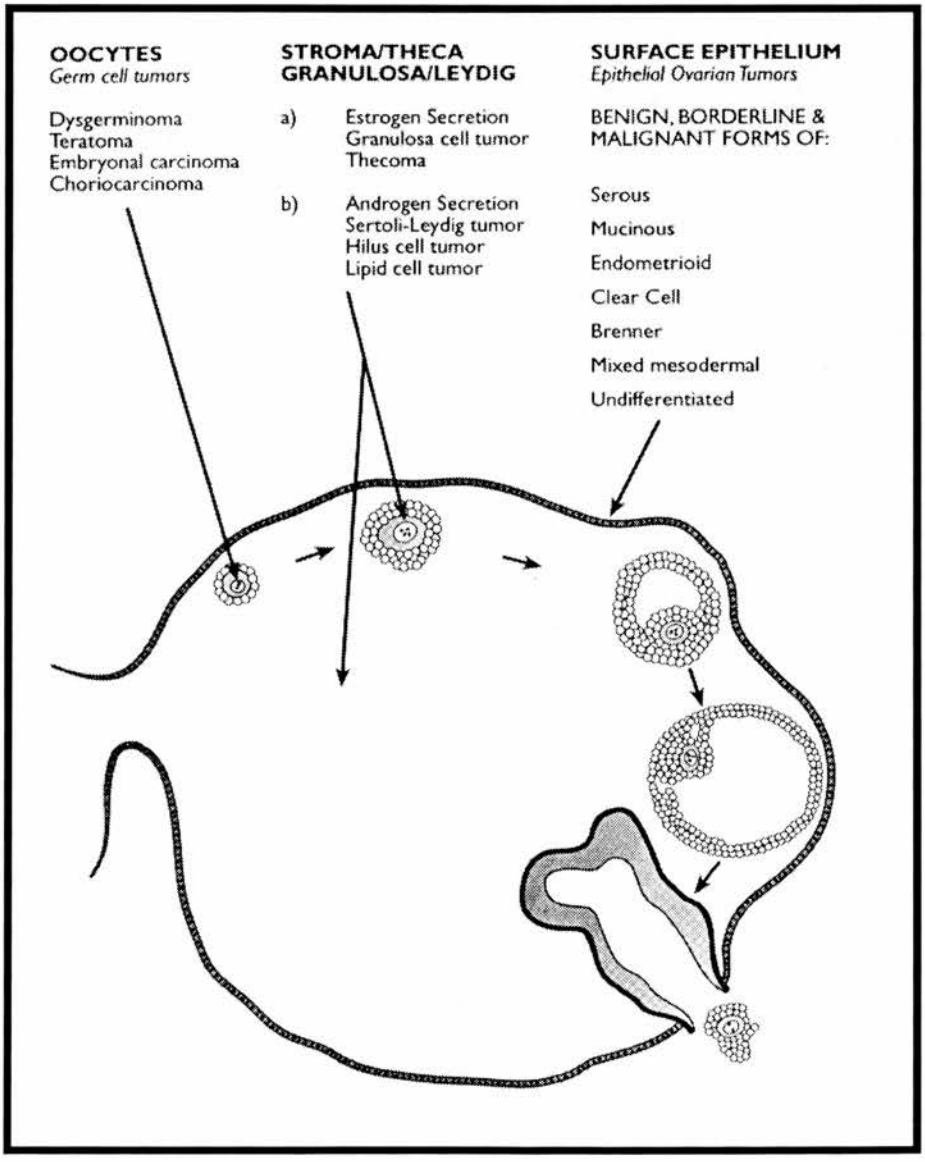
The mesonephric (Wölffian) duct that goes on to connect the gonads to the urethra in the adult male has no way of connecting with the developing ovarian cortex, and so germ cells in the adult female must be shed from the ovarian epithelium rather than transported in the same way as spermatozoa. At week six, a second duct forms from the coelomic epithelium dorsal to the Wölffian(mesonephric) duct. This is the Müllerian (paramesonephric) duct which at the cranial end opens into the coelomic cavity (forming the future fallopian tubes), and at the caudal end fuses with the contralateral Müllerian duct to form the uterine canal. In the female, the Müllerian duct continues to develop into the main genital duct while the Wölffian duct atrophies almost completely. As the ovary develops and migrates caudally, so the Müllerian duct goes on to form the fallopian tubes, the uterine body and the uterine cervix, each with distinctive lining epithelia. Although in continuity with these structures, the vagina develops separately, arising from the urogenital sinus located caudally.

Common epithelial tumours of the ovary (including epithelial ovarian cancer) therefore arise from the ovarian surface epithelium (derived from the primitive coelomic mesothelium), and exhibit a range of histological structures which have appearances similar to other Müllerian derived structures; i.e. serous differentiation (similar to fallopian tube epithelium), endometrioid differentiation (uterine

body endometrium) and mucinous differentiation (endocervical epithelium).

Structures within the ovary not derived from this epithelium produce much rarer tumour types. The mesoderm underlying the primitive coelomic mesothelium generates theca, granulosa, Leydig and Sertoli cells, and all these cell types can give rise to tumours. The primordial germ cells go on to form oocytes and from these can arise a range of germ cells tumours. Dysgerminoma, teratoma (often mature, benign and of parthenogenetic origin in females) and embryonal carcinoma may occur. They will not be discussed further here (see figure 1.1).

**Figure 1.1 Ovarian tumours: Histogenesis**



### 1.2.2 Pathology Of The Common Epithelial Tumours

Epithelial ovarian carcinomas represent over 90% of all ovarian malignant neoplasms. Epithelial neoplasms of the ovary comprise benign tumours, borderline tumours (tumours with low malignant potential), and frank epithelial carcinoma. These three categories of neoplasm usually exhibit Müllerian histogenic differentiation as described above.

#### *1.2.2.1 Benign tumours*

These tumours have an excellent prognosis. The essential cytological features of benign tumours are a single layer of columnar cells, lack of cellular atypia, a normal nucleocytoplasmic ratio, few mitoses, and no evidence of either microinvasion or frank invasion into the underlying stroma. Benign tumours usually exhibit either serous or mucinous differentiation. These tumours are usually cystic and can grow to enormous size.

Half of all serous tumours are benign serous cystadenomas. They are sometimes bilateral, and generally unilocular with a few intracystic papillae which may be either pedunculated with fronds or sessile. The fluid within the cyst is watery (serous). The serous epithelium is well differentiated, and forms a regular single layer of cuboidal cells with centrally placed nuclei lying on loose stroma.

Mucinous cystadenomas constitute 80% of all mucinous tumours. These benign tumours are almost always unilateral. The cysts are multilocular containing a gelatinous mucinous secretion. The epithelium consists of tall columnar epithelial cells with basal nuclei. Sometimes this epithelium is similar to endocervical epithelium consistent with a Müllerian origin, however, more commonly the epithelium is reminiscent of colonic mucinous glandular epithelium with the presence of goblet or Paneth cells, suggesting a tumour of non-Müllerian origin.

Occasionally spillage of mucin-secreting epithelial cell clusters into the peritoneal cavity can result in pseudomyxoma peritonei, a chronic and

often ultimately fatal condition with mucinous secretion filling the peritoneum and causing bowel obstruction without destructive invasion.

Brenner tumours are usually benign ovarian tumours in which there are cords of urothelium embedded in a dense stroma. This tumour is thought to arise from remnants of Wölffian origin (see above), part of the primitive urothelial system.

Endometrioid and clear cell tumours are rarely benign.

#### *1.2.2.2 Borderline tumours (tumours of low malignant potential, LMP)*

These tumours have a good prognosis generally. Borderline tumours are characterised histologically by the presence of pseudostratification of the cells forming the tumour's epithelial lining on a fibrovascular underlying stroma. Nuclear and cellular atypia, increased mitotic activity and detachment and reimplantation of neoplastic cell clusters are all features. However, the pathological diagnosis also requires the absence of neoplastic cell invasion of the underlying stroma. This last feature differentiates borderline tumours from malignant invasive tumours.

Serous LMP tumours, the commonest histological type of borderline tumour, in addition to the above, may also contain psammoma bodies (histological structures which confer a favourable prognosis on serous tumours), and may be multicentric (the clonality of which is currently unclear).

Mucinous LMP tumours are less common than serous LMPs and may be difficult to distinguish from malignant tumours. Again, the majority show intestinal rather than Müllerian differentiation, and pseudomyxoma peritonei may be a complication.

Endometrioid LMPs constitute a fifth of all endometrioid tumours and may be associated with endometriosis (as can clear cell LMPs).

*1.2.2.3 Malignant tumours (epithelial ovarian cancer)*

Frank ovarian adenocarcinomas carry an extremely poor prognosis if disseminated from the primary site (which they do in the majority of cases). The primary tumours often contain solid regions of malignant tissue which can grow rapidly and often exhibit haemorrhagic and necrotic regions. The tumour tends to invade destructively and may breach the ovarian capsule, inducing adhesions locally and spreading into the peritoneal cavity.

The histological hallmarks of malignant tissue are the same as for borderline tumours, with cellular atypia, a high nucleocytoplasmic ratio, and frequent mitoses. There is, however, destructive invasion of the underlying stroma by the malignant adenocarcinoma cells. As part of histopathological diagnosis, an attempt at estimating the degree of differentiation of ovarian adenocarcinomas should be made (Baak et al, 1987). Well differentiated carcinomas demonstrate little cellular atypia and relatively few mitoses ( grade 1). Moderately differentiated carcinomas exhibit retention of the histological differentiation but there is increasing cellular atypia and more frequent mitoses (grade 2). Poorly differentiated carcinomas are more aggressive still, with abundant mitoses and sheets of poorly or undifferentiated cells with often very few features providing clues to the histological category (grade 3). Tumours can entirely lose their histogenic features leaving an undifferentiated adenocarcinoma, which is rapidly growing and behaves aggressively.

One third of all serous tumours are malignant, and serous adenocarcinomas account for just under half of all epithelial ovarian cancers (EOCs). They are frequently bilateral and are often cystic with solid regions within.

Endometrioid tumours are usually malignant, they account for about 15% of all EOCs. They are usually less cystic than serous and mucinous adenocarcinomas. In a quarter of cases endometrioid adenocarcinomas may co-exist with endometrial carcinoma, and have identical

histology. They can also co-exist with, or arise on, a previous site of endometriosis (Heaps et al, 1990)

Mucinous adenocarcinomas account for only one fifth of all mucinous tumours, and represent only about 12% of EOCs. They are frequently cystic and multilocular, may be bilateral, and are often well differentiated when recognised.

Clear cell carcinomas are uncommon (6% of EOCs) and so-called because of the appearance of the cytoplasm after removal of the abundant cytoplasmic glycogen during the specimen preparation process. Like endometrioid carcinoma, they may co-exist with endometriosis.

Undifferentiated EOCs account for about 17% of the total, and tend to behave in an aggressive fashion.

### 1.2.3 The early histological lesion and relationships between benign, borderline and malignant disease.

The FIGO staging system (FIGO Cancer Committee, 1986) (Table 1.1) and the anatomical view of epithelial tumour spread suggests an orderly progression from normal cell through benign tumour to early malignant tumour followed by the breaching of an underlying serosal layer with vascular and lymphatic tumour dissemination, and finally, metastases. However, although such a pathological multistep model may be valid for colorectal cancer (Vogelstein and Kinzler, 1993), the evidence does not favour this for ovarian cancer.

Relatively little work has been done on the features and the nature of the earliest histological lesion in ovarian cancer. In patients who had oophorectomy where ovarian carcinoma was not recognised preoperatively, and who were found to have de novo ovarian carcinoma (i.e. without atypical lesions) on review, tumours were small, ranging from microscopic foci to 7 mm diameter. The tumours were all unilateral, and some were multifocal. They arose from the surface epithelium or from the crypts/inclusion bodies associated. The

non-carcinomatous surface epithelium and inclusion bodies exhibited "severe atypia" in a fifth of cases, suggesting a preneoplastic lesion. Several women went on to develop peritoneal carcinomatosis, suggesting stage III disease essentially from the outset, with fatal outcome. A significant proportion had normal serum CA125 levels (see below) and their tumours would not have been detected radiologically. It is therefore clear that some ovarian cancer are capable of progressing from microscopic ovarian malignant disease straight to potentially fatal stage III disease with no prospect of screening related intervention (Bell et al, 1994).

The presence of multifocality, atypia and inclusion cysts in these very early malignant lesions suggests the possibility of field changes affecting the ovarian epithelium. A case/control study examining in detail the contralateral ovaries from patients with unilateral ovarian carcinoma compared to age-matched controls showed a significantly higher number of inclusion cysts in the unaffected ovaries of ovarian cancer patients with frequent evidence of serous metaplasia within these inclusion cysts. In the ovarian cancer patient group, an age related increase in the number of inclusion cysts was seen, which was not observed in the control group (Mittal et al, 1993). These features suggest an histologically abbreviated route to malignant ovarian cancer.

Over 90% of specimens of mucinous epithelial ovarian cancer contain surrounding benign neoplastic epithelium. In a quarter of cases one can observe a region of transition from benign to malignant tissue (Puls et al, 1992). Such benign epithelia are seen frequently in mucinous carcinomas but relatively infrequently in serous carcinomas, with a suggestion therefore that serous carcinomas may arise de novo more frequently than mucinous carcinomas. A recent analysis (Zheng et al, 1995) demonstrated that approximately 50% of ovarian cystadenocarcinomas contained p53 mutations, whereas there was no evidence of p53 mutation in 37 benign and borderline

epithelial tumours. They next identified malignant tumours with contiguous regions of apparently benign neoplasia and demonstrated that benign looking regions contained the same mutations in p53 as were present in the adjacent malignant regions. These findings suggest that benign neoplasia in the context of cystadenocarcinoma represents a genetically and biologically distinct entity from the solitary benign cystadenoma, and that at a molecular level it is clonally contiguous with the adjacent malignant tumour. Whether these benign regions represent precursors that progress to frank adenocarcinoma or are products of a differentiating effect such as that seen in teratoma is unclear and hinges on further analysis of these regions to demonstrate whether or not they share the full complement of genetic abnormalities found in the adjacent adenocarcinoma. It is of interest that first and second degree relatives of ovarian cancer patients have five times the incidence of benign tumours compared to those without a family history (Bourne et al, 1991).

Endometriosis may well be a premalignant condition. It co-exists with endometrioid cancer in nearly a third of cases; and with clear cell carcinoma in a half of cases, and there are documented cases of endometriosis progressing to epithelial ovarian cancer in some patients taking exogenous oestrogens (Heaps et al, 1990).

In contrast, true serous borderline tumours ( i.e. without evidence of invasion) undergo malignant transformation in only 0.7% of cases (Kurman et al, 1993), suggesting that the pathways to LMP tumours do not form part of the multistep pathway from normal surface epithelium to epithelial ovarian cancer.

These phenotypic lesions do not preclude molecular lesions in phenotypically normal ovarian surface epithelium, and there is evidence that mutant or over-expressed normal p53 is detectable in apparently normal epithelia adjacent to ovarian neoplastic tissue suggesting the possibility of phenotypically normal precursor lesions. To what extent this represents a molecular field change is unknown.

## 1.3 Epithelial Ovarian Cancer: Clinical Management and Prevention

### 1.3.1 Prognostic Factors

Many features of ovarian cancer; surgical, pathological and biological may be associated with survival. It is of importance whether these so-called prognostic factors are truly independent in themselves or simply reflect associations with other prognostic factors. Multivariate statistical methods have been used to answer this question. Such analyses suggest that there are general independent prognostic factors such as younger patient age and good performance status.

Lower FIGO stages (see below), well differentiated tumours (lower tumour grade), low residual tumour volume, and low pre-operative tumour volume are also favourable independent prognostic factors (de-Souza et al, 1992). Serum CA125 (see below) measured three months after the start of chemotherapy correlated well with survival of patients (Sevelda et al, 1989).

Patients with clear cell histology have worse prognosis, specifically in early stage disease (Guthrie et al, 1984; O'Brien et al, 1993).

Flow cytometric analysis of tumoral DNA content can indicate the presence of aneuploid tumour populations and is a significant adverse independent prognostic factor (Friedlander et al, 1984; Friedlander et al, 1988).

However, it is not enough to simply derive prognostic indices from multivariate analysis, these factors must then be applied in prospective studies to test their usefulness in practice.

The use of molecular biological analysis will hopefully yield tumoral markers which have prognostic utility. Tumoural oestrogen and progesterone receptor content, erb-b2 (HER2/neu) and epidermal growth factor receptor levels are promising candidates. The data examining the role and independence of p53 abnormality as a prognostic factor in ovarian cancer is conflicting, with some investigations showing no association with p53 abnormalities (Kohler et al, 1993; Niwa et al, 1994; Sheridan et al, 1994; Frank et al, 1994); and

others showing that p53 status functions as an independent prognostic factor (Henriksen et al, 1994; Klemi et al, 1994; Levesque et al, 1995; Vanderzee et al, 1995). P53 mutation is reported to be significantly associated with serous histology (Eccles et al, 1992; Niwa et al, 1994). Recent data suggests that those ovarian cancers which have a normal p53 gene by sequence analysis are more likely to respond to chemotherapy than those with p53 mutation (Al-Azraqi et al, 1995).

The use of some of these markers in combination (PR, DNA ploidy, S-phase fraction and Ki67, a marker of cell cycling) has been shown to have similar (though prognostically independent) power to FIGO stage in correlation with progression free interval and overall survival of ovarian cancer patients (Kaufmann et al, 1995).

### 1.3.2 Staging And Primary Surgical Treatment

The most powerful determinant of prognosis for epithelial ovarian cancer is the extent to which the malignant tumour has disseminated from its primary site at the time of diagnosis. In order to stage the patient adequately, thorough surgical evaluation by exploratory laparotomy needs to be undertaken. Removal of all tumour within the pelvis and peritoneal cavity offers the patient the best chance of ultimate control of their disease. Thus definitive surgery is employed with abdominal exploration in a procedure that constitutes primary treatment as well as indicating the patient's prognosis.

This rigorous approach to surgical evaluation has resulted in the widespread adoption of the FIGO staging system (FIGO Cancer Committee, 1986) for ovarian cancer, where ascending stage correlates with worsening prognosis (Table 1.1). Accurate staging of the patient is crucial as it determines subsequent therapy, which will contribute to outcome.

If there is obvious evidence of tumour spread, debulking of residual tumour masses (up to a maximum diameter of 10 cm) to under 2 cm maximum diameter should be undertaken as there is evidence that

this results in better response and survival with subsequent chemo- or radio- therapy.

**Table 1.1 The FIGO staging classification**

| Stage | Description |
|---|---|
| Stage I | Growth limited to ovary |
| Ia | One ovary involved, no ascites, capsule intact, no tumour on the external surface |
| Ib | Both ovaries involved, no ascites, capsule intact, no tumour on the external surface |
| Ic | tumour on ovarian surface, rupture of capsule, malignant ascites present/positive peritoneal washings |
| Stage II | Growth limited to the pelvis |
| IIa | Extension to gynaecological adnexae; uterus/fallopian tubes |
| IIb | Extension to other pelvic tissues |
| IIc | Stage IIa or Iib with tumour on the surface of both ovaries, rupture of the capsule, malignant ascites, or positive peritoneal washings |
| Stage III | Growth extending to peritoneal cavity- including peritoneal surface seedlings, omentum, or serosal surfaces of viscerae |
| IIIa | Grossly limited to the true pelviswith negative lymph nodes, but microscopic seedling of the abdominal peritoneal surface |
| IIIb | Tumour involves one or both ovaries with histologically proven implants of the abdominal peritoneal surfaces, none > 2cm |
| IIIc | Abdominal implants >2cm diameter and/or positive retroperitoneal lymph nodes |
| Stage IV | Metastases to distant sites; pleural effusions must be cytologically positive |

### 1.3.3 Chemotherapy

Patients who do not have early-stage ovarian cancer with good prognostic features (Stage Ic, Stage I with moderately/poorly differentiated carcinoma, Stage II-IV disease, and clear cell carcinoma irrespective of stage) should receive chemotherapy as first-line adjuvant therapy. "Advanced disease" can be considered according to the consequences of laparotomy: whether or not debulking was optimal. "Optimal" or "small volume disease" in a European context is defined as maximum residual diameter 2 cm after surgery. Above

these diameters patients are regarded as having "sub-optimally debulked" or "large volume" disease.

*1.3.3.1 Platinum analogues*

Standard chemotherapy generally consists of platinum containing regimens, usually with an alkylating agent.

The best results suggest that using multimodality therapy, the five year survival of advanced (stage III-IV) epithelial ovarian cancer can be improved from approximately 12% to approximately 30%.

Platinum analogues (e.g., cis-diamino-dichloro-platinum, CDDP) function as cytotoxic drugs by binding to amino or hydroxyl groups of nucleoside bases and forming DNA intra-strand and inter-strand crosslinks. This DNA crosslinking results in interference with replication/repair mechanisms.

In those with small volume disease, a significant survival advantage was seen for the use of platinum (Neijt et al, 1994).

Abrogation of platinum drug resistance in vitro has been observed when tamoxifen is added concurrently. The mechanism underlying this observation is obscure, and is under intensive investigation (McClay et al, 1992; Nakata et al, 1995). However, clinical studies do not support the use of a platinum/tamoxifen at the moment (Schwartz et al, 1989).

The combination of platinum with interferon based on in-vitro evidence for synergy between platinum and interferon in ovarian cancer (Nehme et al, 1994) to improve response rates has produced only modest clinical benefit (Markman et al, 1992; Ferrari et al, 1994).

Results from a randomised study attempting to reduce the toxicity of cis-platinum using glutathione (an intracellular free radical scavenger) suggest that more full dose cycles can be given on time with significant reduction in nephrotoxicity. The incidence of nausea, vomiting and neuropathy were significantly reduced in the glutathione group. The response rate was not reduced, suggesting that glutathione improved the therapeutic index of cis-platinum (Smyth et al, 1995).

### *1.3.3.2 Taxanes*

Taxanes are natural (taxol) and semi-synthetic (taxotere) diterpenoids. Taxol (Paclitaxel) was discovered in 1971 when it was isolated from the bark of the western yew tree (Taxus brevifolia). Taxoids induce tubulin polymerisation, and also prevent the depolymerisation of microtubules resulting in stable, non-functional microtubules disrupting cellular organisation and producing effective mitotic arrest. The American gynecologic oncology group (GOG protocol 111) have recently reported mature data from a randomised study of sub-optimally debulked ovarian cancer patients, comparing standard first line chemotherapy (cyclophosphamide/cis-platinum, CP) with taxol /cis-platinum (TP). Both the complete and partial response rates were significantly increased in the taxol/platinum arm. Median survival was increased from 2 years (cyclophosphamide/cis-platinum) to 3 years for the taxol/platinum arm (McGuire et al, 1995). The impact of this study has been to impose platinum/taxol as the gold standard in adjuvant chemotherapeutic treatment of ovarian cancer.

### *1.3.3.3 Drug resistance*

It is generally accepted that chemotherapy fails to eradicate cancer due to a combination of intrinsic and acquired drug resistance. Physiological host factors may alter drug disposition within the body resulting in a reduction of the amount of active drug getting to the tumour (reduced activation, enhanced excretion and breakdown), but resistance is determined more by tumour cellular factors. In terms of ontogeny, tumour cells represent extremely old human cells (having ceaselessly divided over a long period of time), and have acquired many genetic lesions that allow them to escape normal growth regulation. They can evoke mechanisms to circumvent attempts to control their growth. One manifestation of this survival pathway redundancy is drug resistance.

Tumour cells may limit drug accumulation by influx pump inhibition or by efflux pump augmentation(multi-drug resistance, p-glycoprotein).

Intracellular inactivators of drugs may be upregulated by tumour cells. Such inactivators include scavenger antioxidants such as glutathione for example, which binds intracellular platinum among other drugs and may prevent DNA adduct formation.

Damage to genes which maintain the stability and integrity of the genome allow the cells to deregulate their tight control over the genome with rapid and often bizarre changes mediated by selective pressure from a hostile environment. p53 abnormality and mutation of mismatch repair genes resulting in the 'promutator phenotype' may contribute to a phenotype tolerant to the adverse environment created by the cytotoxic milieu. This may manifest in the ability to circumvent apoptosis (programmed cell death) by mutations acquired in proteins involved in the pro-apoptotic pathway.

Additionally, tumour cells may be able to develop DNA repair mechanisms that are more efficient than normal, counteracting the genotoxic effects of cytotoxic chemotherapy, and hence contributing to resistance to DNA damaging drugs. An overview of DNA repair mechanisms is beyond the scope of this thesis: many enzymes are involved in the process of damage recognition, nucleotide excision, re-synthesis of the damaged region with fidelity, and ligation to re-establish the continuity of chromatin.

Drug resistance may also be associated with alteration of the topoisomerase pathway (either by mutation or altered expression). This enzyme is involved in cleavage of DNA, passage of a DNA strand through the cleaved segment and resealing of the DNA. Type I topoisomerases cleave only one strand; type II cleave both strands.

## 1.4 Molecular Genetics of Epithelial Ovarian Cancer

### 1.4.1 Molecular epidemiology and genetic linkage

#### *1.4.1.1 Molecular epidemiology*

The molecular study of genes involved in carcinogenesis has opened new avenues into epidemiology. It is possible by analysis of genes thought to be important in the aetiology of a particular tumour type to make inferences about causation, the relative balance of inherited to somatic events and whether the somatic events are due to environmental or spontaneous processes. For instance, p53 is one of the genes most commonly mutated in cancer. Molecular epidemiology of the p53 gene in hepatocellular carcinoma where aflatoxin has been the underlying carcinogen reveals a high frequency of transversions (mutation where purines are replaced by pyrimidines or vice versa) of the guanine to thymine (G to T) type at a specific site of the gene's sequence (codon 249) (Bressac et al, 1991). The presence of transversions at such sites represent events related to exogenous carcinogens, whereas transitions (conversion of pyrimidine to pyrimidine, or purine to purine) are more likely to be due to errors of fidelity during DNA replication, which may be a spontaneous process.

p53 is frequently mutated in epithelial ovarian cancer; however similar analysis of the p53 gene in ovarian cancer revealed no such transversion "hot-spots"; mutation of transition type were predominant (72% of all mutation events detected) and dispersed throughout the gene(predominantly exons 5 to 8) (Kohler et al, 1993). This predominance of transition mutations suggests that the impact of environmental carcinogens to the development of ovarian cancer may be minimal. Although this data is generally supported by the epidemiological evidence available, it is important to remember that environmental carcinogens may mediate their action on other genes involved at other stages of ovarian cancer development.

*1.4.1.2 Inherited predisposition: overview*

Clearly defined predisposing /hereditary factors account for only about 5% of patients with ovarian cancer, as judged by familial clustering. In most of these familial cases, the aetiology is probably multifactorial, consisting of both environmental and genetic elements. Women with one first degree relative affected run a 5% lifetime ovarian cancer risk (1 in 20). The risk associated with having 2 or more affected first degree relatives is 7% (1 in 15).

Those with definable autosomal dominant syndromes run a much higher risk (40-50%) of developing ovarian cancer, but these individuals constitute only about 3% of women with two affected first degree relatives (Narod, 1994).

Three such hereditary syndromes have been defined, and patients with these tend to be younger(usually 10 or more years less than the median) and have bilateral and multifocal tumours more commonly. Major international efforts have been and are in progress to identify the spectrum of genes involved in these syndromes.

Hereditary site-specific ovarian cancer is rare and in the majority linked to BRCA1 (Steichen-Gersdorf et al, 1994, a putative tumour suppressor gene located on chromosome 17q (see "BRCA1" below).

Hereditary breast/ovarian cancer is diagnosed in the presence of familial clustering of breast and ovarian cancer. Hereditary ovarian cancer is most likely to occur as part of this syndrome. Again, most of these are BRCA1 linked (Easton et al, 1993a).

The type II Lynch syndrome incorporates hereditary non-polyposis colorectal cancer (Lynch syndrome I) with gastrointestinal (gastric, small intestinal, pancreatic), gynaecological (endometrial and ovarian), urological or breast cancer (Lynch et al, 1985a; Lynch et al, 1985b; Lynch and Lynch, 1993). This syndrome is linked to a family of DNA mismatch repair genes, disruption of which result in "pro-mutator" effects on the genome, often observed as a high incidence of microsatellite instability. These genes have been designated hMSH2

(Fishel et al, 1993), hMLH1 (Kolodner et al, 1995), hPMS1 and hPMS2 (Nicolaides et al, 1994).

### *1.4.1.3 BRCA1 and ovarian cancer*

The BRCA1 gene is large, extending over 100 Kb of genomic DNA, with 24 exons. It encodes a protein of 1863 amino acids that is expressed in many tissues during development, including thymus, breast and testis (Miki et al, 1994). It has a ring finger domain consistent with DNA binding and can localise to the nucleus but there is, as yet, no proof that it acts as a transcription factor. It is strongly expressed in the epithelial cell layer of the adult ovary but expression appears to be reduced in malignant cells (Thompson et al, 1995). BRCA1 is expressed in rapidly proliferating cell types undergoing differentiation, and its expression is induced in oophorectomised animals after treatment with 17 beta-oestradiol and progesterone (Marquis et al, 1995). An inhibitory role for BRCA1 has now been determined in breast and ovarian cancer cell lines. Transfection of these cell lines with plasmid expressing wild-type BRCA1 inhibits growth, although this is not the case for colon or lung cancer cell lines, demonstrating a tissue specific effect. Though somatic mutation of BRCA1 is evidently rare, loss of the wild type allele has been found in many studies of ovarian and other tumours occurring in carriers of a germ-line mutation and, as noted above, LOH on 17q, including the BRCA 1 locus, is a common event in ovarian cancer (Takahashi et al, 1995; Cornelis et al, 1995). BRCA1 therefore has most of the characteristics of a tumour suppressor gene.

The aggregate mutation frequency of BRCA1 in Western populations is between 0.03% and 0.1% (Easton et al, 1993). Ashkenazy Jews have a particular mutation (185 del AG) at a frequency approaching 1% (Struewing et al, 1995).

Germ line mutations are found at many different positions within BRCA1. The majority of described mutations are nonsense or frame-shift, generating a truncated protein. About 25% appear to be

regulatory changes, inhibiting transcription of one allele. Overall, there is about a three-fold greater risk of breast than ovarian cancer among carriers of BRCA1 mutations but the data suggest that these relative risks are not uniform in different families (Easton et al, 1993b). Findings from a British study (Gayther et al, 1995) indicate that mutations closer to the 5' end of the gene carry a higher risk of ovarian cancer. Interestingly, although transfection of a 3'BRCA1 mutant (1835stop) does not inhibit the growth of breast cancer, it still does inhibit the growth of ovarian cancer cells (Holt et al, 1996). Furthermore, this 3' mutation appears to to map to BRCA1's putative granin consensus sequence (Jensen et al, 1996), and this suggests a mechanism for mutational differences involved in tumours of different tissue origins.

Other explanations for non-uniform distribution of breast and ovarian cancer risks in BRCA1 families include the use of exogenous hormones. Prolonged use of the combined oral contraceptive "pill" has been shown to reduce the risk of ovarian cancer (Parazzini et al, 1991) (see above). Co-inheritance of modifying genes may influence the "BRCA1 phenotype". The presence of rare alleles at the HRAS1 VNTR locus (1 Kb downstream of the HRAS1 proto-oncogene) conferred a 2.1 fold increased risk for ovarian cancer in BRCA-1 carriers compared with BRCA1 carriers harbouring common alleles only. Susceptibility to breast cancer was not altered by the presence of the rare alleles in BRCA1 carriers and so the allele appears to specifically modify the ovarian cancer risk in this inherited syndrome (Phelan et al, 1996).

The lifetime risk of ovarian cancer in a carrier of a BRCA1 mutation is estimated at between 20 and 80% (Easton et al, 1993). The tumours rarely present under age 35 and though, in most studies, the age of onset of the disease has tended to be younger than for sporadic cases, there is some disagreement as to the value of age of onset as a marker for genetic predisposition; Screening is available for high risk

individuals but its value is unproven. (see "prospects for prevention" above)

*1.4.1.4 BRCA2 and ovarian cancer*

It appears that a significant proportion of inherited breast cancer may be attributable to a susceptibility gene located on chromosome 13q12-q13 which has been called BRCA2 (Wooster et al, 1994). The initial impression is that it does not confer substantial increase in the risk of ovarian cancer, however, 13q LOH in the presence of normally expressed Rb-1 product suggests that it may have some role in ovarian cancer. The gene was cloned late in 1995 (Wooster et al, 1995) and consists of an 11-12 kb messenger RNA encoded by a 27 - exon gene spanning 70 kB of DNA. It encodes a 3418 aa protein of about 350 kd. The gene has no obvious strong homologies; the mouse and human genes share only about 55-60% homology. However, it contains 8 copies of a highly conserved repeat element dispersed throughout the gene, whose function is as yet unclear. Over 50 mutations have been described in BRCA2, nearly all of which are frame-shift or nonsense, and no mutational hotspots have been identified (Couch et al, 1996; Neuhausen et al, 1996; Phelan et al, 1996; Wooster et al, 1995). In one of these reports, only 2 of 17 non-BRCA1-linked breast/ovarian cancer families contained a BRCA2 mutation (Couch et al, 1996). These findings suggest that perhaps a greater than previously accounted for proportion of inherited ovarian cancer may be linked to a third susceptibility locus.

It seems likely that the absolute lifetime risk of ovarian (as distinct from breast) cancer will be considerably lower than for BRCA1 mutation carriers but it may well be high enough to raise questions of screening and/or preventive surgery (Ford and Easton, 1995).

*1.4.1.5 Other recessive genes in ovarian cancer*

Similarly, over the next few years our understanding of the clinical consequences of RER gene mutations is bound to grow and it may emerge that for some individuals, perhaps depending on the precise

mutation present or the details of the family history - or both - specific action will be appropriate in relation to the ovarian cancer risk.

The possibility of a separate ovarian cancer gene, distinct from those mentioned above, responsible for some familial cases has not been formally ruled out but epidemiological studies indicate that if it exists at all, it cannot account for more than a small proportion of cases.

Relatively common lower penetrance gene aberrations (notably of ATM, mutations in which cause Ataxia Telangiectasia in the homozygous state) (Savitsky et al, 1995) have been invoked to explain a significant proportion of breast and some other cancers. However, there is no evidence at present to suggest that they are important in ovarian cancer.

### 1.4.2 Structural Genetic Alterations in Ovarian Cancer

#### *1.4.2.1 Cytogenetics*

As would be expected from a disorder caused by lesions to the somatic genome, gross chromosomal abnormalities are evident in ovarian cancer. The methodological simplification associated with using malignant cells from ascites specimens for cytogenetic analysis have generally skewed the data towards more advanced ovarian malignancies, with evidence of the bizarre end-products of extreme genomic instability. A problem associated with this approach is how to ascribe causality (via genetic lesions underlying the chromosomal aberrations) to the observed cytogenetic lesion, and one can try to describe frequent non-random rearrangements such as those described for haematological tumours. Often, however, the complexity of the rearrangements precludes such definitive comment and therefore one has to use molecular genetics, using both structural and functional studies to attempt to answer this question.

Among those cytogenetic lesions that are frequently reported are those involving chromosomes 1,3,6,9,11,12,17,19 and X (Jenkins et al, 1993; Bello et al, 1990; Pejovic et al, 1992).

In ovarian cancer, cytogenetic analysis has demonstrated partial deletions of chromosome 11 affecting both long and short arms (Jenkins et al, 1993; Bello et al, 1990; Pejovic et al, 1992).

In-situ hybridisation methods provide refinement to the cytogenetic data. Iwabuchi and colleagues (Iwabuchi et al, 1995) utilised comparative genomic hybridisation (CGH) in a series of 56 ovarian tumours including 44 ovarian cancers. Copy number abnormalities were more frequent in high grade cancers and not present in any of the benign tumours. In only 31% of cases overall was there concordance between LOH for a chromosome arm and physical deletion as judged by CGH. This finding is highly instructive in demonstrating that flanking regions which do not show LOH must contribute to the discrepancy between published LOH and CGH analyses of the same regions and demonstrates that concordance occurs mainly where contiguous LOH is long enough to approach the limits of resolution defined by CGH. Of the most frequent 18 copy number abnormalities detected, 13 were instances of increased copy number. The 6 most frequent observable CGH reductions of copy number were 17 pter-q21 ( 46%; containing p53), 16q (38%), 8p21-p23 (27%), Xp (27%) and whole 19 (27%).

### *1.4.2.2 Loss of Heterozygosity*

Molecular analysis of sporadic ovarian cancer has absorbed a large research effort over the last 10 years. A genome mapping approach using polymorphic DNA probes has been used to identify regions of possible deletion (often based on prior cytogenetic evidence of specific frequent chromosomal disruption). This approach is based on the hypothesis proposed by Knudson (Knudson, 1971) extrapolated from classical genetics observations in hereditary retinoblastoma. Knudson studied 48 cases of retinoblastoma and observed 3 familial cases with multifocal disease and earlier ages of onset. This data when analysed produced empirical data which were consistent with the hypothesis that two mutational events were required for either familial or

sporadic retinoblastoma. Later, these hits were postulated to be associated with loss of function which in turn uncovered the neoplastic state. This "two-hit" hypothesis has become a paradigm for the characterisation of tumour suppressor genes, and such "allele loss" studies presume that one "hit" is due to a deletion involving one allele, the other hit being a more subtle event such as a point mutation. As mentioned, this concept can be extended to anonymous polymorphic loci in the vicinity of observed frequent non-random sites of cytogenetic disruption as a way of identifying potential sites of tumour-suppressor genes by loss of heterozygosity (LOH) in tumour DNA as compared with constitutive genetic material.

LOH does not provide direct proof of a tumour suppressor gene. However, specific chromosomal regions demonstrating very high levels of LOH flanked by regions of much lower loss provide attractive candidate regions for a positional cloning approach.

Many ambiguities and difficulties exist in the interpretation of LOH data. This is reflected in the sometimes strikingly discordant results published by different groups examining the same chromosomal regions. Firstly, published studies often contain small numbers of tumours. Even in larger studies bias may still be introduced by unrepresentative populations of early or advanced stage, low or high grade, and altered proportions of histological type tumours. Furthermore, surgical procedures may not be standardised from centre to centre e.g., a tumour labelled as 'early' may in fact be advanced due to inadequate surgical sampling.

Secondly, technical considerations may create problems. The extent to which tumour is dissected free of contaminating normal tissue is important since increasing amounts of normal contamination will reduce the observed allele imbalance and thus reduce the final LOH rate at a particular locus. The cut-off value for meaningful allele imbalance varies between studies from 30% to 50% reduction in intensity comparing the ratio in normal tissue with the ratio of alleles

in tumour tissue. Clearly this will have an impact on the reported LOH rate. Furthermore, the frequency of LOH required at any locus in order to ascribe significance to the locus is not defined. Some workers have suggested that LOH occurring above a baseline level of 35% represents potentially causative alterations, and frequencies below this represent secondary phenomena (background noise) associated with generalised genomic instability (Cliby et al, 1993), but these cut-offs are arbitrary and vary widely.

Thirdly, variation in reported LOH rates on chromosome arms may be due to the informativeness and density of the polymorphic markers used. There is increasing evidence that LOH may be restricted to narrow sub-chromosomal regions, and thus a single polymorphic marker per chromosome arm such as employed in many allelotype studies may yield unrepresentative information.

The finding of statistical associations with clinicopathological features may suggest possible functions of such regions (Eccles et al, 1992). One major drawback of this type of analysis is that many random allele losses may occur in advanced sporadic tumours which may have no causal relationship with the neoplastic process, but merely represent consequences of primary genome instability associated with cancer.

#### *1.4.2.3 Loss of heterozygosity in ovarian cancer*

To date, there have been over 53 LOH studies looking at epithelial ovarian cancer and assessing allele loss at loci of every chromosome arm. These studies are summarised as a table of chromosomal arms exhibiting significant LOH (>35%) in ovarian cancer (Table 1.2), representing 1996 updates of a review written in early 1994 (Shelling et al, 1995). This table does not take into account the data generated in this thesis.

Although the term LOH is used interchangeably with allele imbalance in this work, it must be borne in mind that imbalance can also be a consequence of allele specific amplification and need not necessarily imply deletion of a region of DNA. Furthermore, amplification of a

region of DNA is not mutually exclusive with loss of function at a tumour suppressor locus; loss of a chromosome or subchromosomal region may occur with reduplication of the other allele/chromosome, and amplification of a region of DNA is not necessarily associated with gain of function if accompanying inactivating mutations are involved.

**Table 1.2 LOH rates of chromosome arms in epithelial ovarian cancer**

| Chromosome arm | Allele loss | % LOH |
|---|---|---|
| 17p | 407/652 | 62% |
| 17q | 370/655 | 56% |
| 22q | 111/224 | 49% |
| 18q | 60/142 | 42% |
| 9q | 45/109 | 41% |
| 9p | 104/259 | 40% |
| Xp | 38/94 | 40% |
| 6q | 111/280 | 40% |
| 5q | 74/183 | 40% |
| 13q | 112/286 | 39% |
| 19p | 46/127 | 36% |
| 21q | 17/49 | 35% |
| 6p | 60/171 | 35% |
| 11p | 151/448 | 34% |

An example of the curious relationships between LOH and gene mutation is found for BRCA 1, the recently cloned familial ovarian/breast cancer gene. This was initially expected to exhibit profound effects in the sporadic counterparts of the disease. It came as some surprise therefore when it was reported that in an apparently sporadic series of 12 ovarian and 32 breast cancers with LOH found using tightly linked and intra-genic microsatellites, only four had mutations of the gene, and in these four, the mutations were found in the germline - suggesting previously undetected familial cases (Futreal et al, 1994). Subsequently a further report (Merajver et al, 1995) demonstrated that of 47 ovarian cancers with LOH at one or both of two polymorphic CA repeat microsatellites intra-genic to the BRCA 1 gene, 4 exhibited evidence of somatic mutation by single strand conformational polymorphism analysis (SSCP). However, this still

leaves 90% of the tumours with LOH with no obvious BRCA 1 mutation. It may be that all these LOH examples will ultimately be accounted for by more thorough analysis of the BRCA 1 region including flanking regulatory regions. Alternatively this discrepancy points to a second gene involved in sporadic cancers of ovary and breast in this region.

Since only a few loci have been studied on each chromosomal arm, further analysis is bound to yield more regions of LOH which have not been delineated thus far, and many chromosomal arms will be subject to interstitial loss rather than whole arm or whole chromosome loss which are particularly common for chromosome 17 in ovarian cancer (Foulkes et al, 1993; Tavassoli et al, 1993), and likely to be related to the presence of the p53 gene on this chromosome.

### 1.4.3 Molecular pathology of ovarian cancer

Ovarian cancer LOH studies have identified frequent losses on chromosomes containing identified tumour suppressor genes such as p53 on chromosome 17p, RB1 and BRCA2 on chromosome 13q, BRCA1 on 17 q21 and DCC on chromosome 18q. Only p53 has demonstrated frequent somatic mutation consistent with the LOH findings. For other chromosomes, the rate of detectable somatic mutation is discordantly low compared with the observed high rate of LOH. This suggests either that these are not the involved suppressor genes, or that there is a preferentially high rate of functional mutation which involves non-coding (e.g. regulatory regions), or that LOH analysis reflects non-significant random "downstream ripples" associated with generalised genomic instability rather than reflecting dysfunction of a "prime-mover" (a TSG). This underpins the need for LOH studies to be supported by functional studies to confirm the relevance of these changes.

*1.4.3.1 p53 in ovarian cancer*

p53, a multifunctional protein involved in cell-cycle control and genome stability is one of the most commonly altered proteins in cancer(Reviewed in Carson and Lois, 1995; Elledge and Lee, 1995; Haffner and Oren, 1995).

The p53 gene is composed of 11 exons, the first of which is non-coding and localised 8 to 10 Kb upstream of exons 2 through 11 on the short arm of human chromosome 17 . There are five conserved domains within the the coding regions of the gene which contain 80% - 90% of described functional p53 mutations. p53 structure is not similar to any other known DNA binding proteins. Two antiparallel beta sheets anchor peptide loops which directly contact DNA and also stabilise the shape of the molecule. p53 monomers assemble into tight dimer pairs, then symmetrical tetramers held together through multiple molecular interactions. The carboxyl end of the p53 protein can also bind to broken DNA ends. p53 has a sequence-specific transactivator domain located in the central 'core' of the protein, comprising residues 90-290.

Normal or wild type P53 is involved in several downstream functions: DNA damage detection, apoptosis and growth arrest. p53 protein has the capacity to detect and bind sites of primary DNA damage (in the form of insertion/deletion mismatches or broken DNA ends). This functional ability to detect DNA damage maps to the carboxyl terminus of the protein forming high affinity complexes with the damaged DNA site, and providing a focus for the subsequent assembly of proteins involved in DNA repair. One of these potential partners is a factor implicated in DNA excision repair known as ERCC3. Binding to DNA has also been shown to protect p53 from rapid proteolysis, which is consistent with the observed stabilisation and accumulation of p53 protein in DNA-damaged cells.

p53 protein can also mediate cell death by apoptosis. This morphologically distinct form of cell death is an active response to cell stimuli. Cell volume shrinks, chromatin condenses, and the nucleus fragments. Phagocytes ingest and destroy apoptotic cells without

provoking inflammation. p53 mediated growth arrest or apoptosis depends partly of the state of cellular activation. Conflicting growth regulatory signals, sustained p53 synthesis due to extensive un-repaired DNA damage, or p53 activation after irreversible commitment to replication can drive a cell to apoptosis. Apoptosis can also occur without the presence of p53 via a steroid mediated pathway.
The retinoblastoma protein (pRb) family are implicated in the relationship between p53 mediated growth arrest and apoptosis. pRb is normally required for imposing a p53 mediated G1 arrest. If pRb is functionally inactive, either physiologically or pathologically, the growth-inhibitory activity of wild-type p53 is converted into an apoptotic fate.
As mentioned above, DNA damage stimulates the production of the p53 protein, in turn transactivating genes such as p21 and GADD45 via p53's central core domain. Transcriptional activity is mediated by a domain located at the amino end of the p53 protein. GADD45 is involved with growth arrest in the presence of DNA damage. p21, however, inhibits the activities of cyclin-dependent kinase enzymes which are responsible for pushing the cell through its cycle for division. This allows for cell arrest at the G1/S phase junction and time for DNA repair. On restoration of DNA integrity, proliferation resumes. Both GADD45 and p21 interact with and inhibit PCNA (proliferating cell nuclear antigen), the function of which is to stimulate DNA synthesis allowing co-ordination of two separate processes; cell cycling/division and DNA synthesis.

Functional p53 mutations usually affect trans-activation domains. More that 90% of the substitution mutations reported so far in malignant tumours are clustered between exons 5 and 8 and most are localised in the evolutionarily conserved regions.
Most p53 mutants fall into one of two classes: DNA contact mutants or structural mutants. DNA contact mutants of p53 are unable to bind

specific DNA target sequences because of substitution of crucial DNA contact residues, including mutational hot spots, such as arginine-248 and arginine-273. Structural mutants, on the other hand, lose specific DNA binding capacity because of abnormal conformation of the protein. Central core mutations are the main mutations affecting trans-activation.

It has been suggested that greater than 60% loss of p53 function is needed to result in a tendency towards neoplastic transformation or growth.

The presence of transversions (mutation where purines are replaced by pyrimidines or vice versa) represent events related to exogenous carcinogens, whereas transitions (conversion of pyrimidine to pyrimidine, or purine to purine) are more likely to be due to errors of fidelity during DNA replication, which may be a spontaneous process.

p53 is frequently mutated in epithelial ovarian cancer and its sequence analysis reveals no transversion "hot-spots"; mutation of transition type were predominant (72% of all mutation events detected) and dispersed throughout the gene(predominantly exons 5 to 8 (Kohler et al, 1993). This predominance of transition mutations suggests that the impact of environmental carcinogens to the development of ovarian cancer may be minimal.

The role and independence of p53 abnormality as a prognostic factor in ovarian cancer is conflicting, with some investigations showing no association with p53 abnormalities (Frank et al, 1994; Kohler et al, 1993; Niwa et al, 1994; Sheridan et al, 1994) and others showing that p53 status functions as an independent prognostic factor (Henriksen et al, 1994; Klemi et al, 1994; Levesque et al, 1995; Vanderzee et al, 1995). p53 mutation is reported to be significantly associated with serous histology (Eccles et al, 1992; Niwa et al, 1994). Recent data suggests that those ovarian cancers which have a normal p53 gene by sequence analysis are more likely to respond to chemotherapy than those with p53 mutation (Al-Azraqi et al, 1995). Recently, a p53 polymorphism

designated p53PIN3 has been identified; homozygotes were 9 times more frequent in a cohort of ovarian cancer patients than in a control group. The polymorphism is due to a 16bp insertional tandem repeat in intron 3 of p53 (Wang et al, 1996). The underlying basis of this association is not yet understood.

### *1.4.3.2 Ovarian cancer and the cell cycle*

Unregulated growth as a result of unchecked progression through the cell cycle is one of the defining features of malignancy. In recent years there have been great advances in our understanding of the subcellular machinery responsible for controlling the cell cycle and its relevance to cancer (Clurman et al, 1995; Kamb et al, 1995; Marx et al, 1994).

At the heart of the cell cycle "engine" are phosphorylating enzymes, cyclin-dependent kinases (CDKs) which associate as complexes with activating holoenzymes (cyclins) and are responsible for driving the cell through each of the checkpoints G1/S, S/G2 and G2/M (Nigg et al, 1995; Morgan et al, 1995). One of the major substrates through which CDKs mediate their effects at the major G1/S checkpoint is the retinoblastoma protein Rb. Rb forms a complex with E2F, a promiscuous transcription factor. When Rb is phosphorylated by CDKs, E2F is released to mediate co-ordinated transcription of genes required for entry into S phase (Weinberg et al, 1995; Cobrinik et al, 1992).

The CDKs are regulated by specific inhibitors (CDKIs) which suppress cycling by binding either to cyclin/CDK complexes (an activity exemplified by the Cip/Kip group of proteins; p21, p27, p57) or to monomeric CDKs (e.g. the INK4 group of CDKs p15, p16, p18, p19).

The latter are more specific since they block the actions of the D-cyclins only. Upstream regulation of the CDKIs is important in signal-mediated control of the cell cycle. For example, p21 is activated by p53 in response to DNA damage while p27 and p15 are induced by

TGF-beta (Toyoshima et al, 1994). Recently, some evidence has been presented that oestradiol can stimulate cyclin/Cdks in ovarian cancer cell lines (Ahamed et al, 1996). Oestradiol has also been shown to stimulate BRCA1 expression in an ovarian cancer cell line (Romagnolo et al, 1996).

The role of these genes in ovarian cancer is starting to become apparent. Deletion (even homozygous deletion) of p16 is found rather frequently in several types of cancer including ovarian cancer - both primary tumours and cell lines. LOH studies show frequent disruption of the p16 region on chromosome 9p but this is rarely accompanied by mutation of the retained p16. It has been postulated that deletion events at this locus are more powerfully tumorigenic (and therefore selected for) because they also involve the adjacent p15 gene (Schultz et al, 1995; Nobori et al, 1994).

As discussed earlier, amplification at 11q13, though less common than in breast cancer, does occur in ovarian tumours and is usually associated with over-expression of cyclin D1 (Alama et al, 1996; Zuckerberg et al, 1995).

Over-expression of MDM2, which has been described as a mechanism for inactivation of p53, for example in sarcomas (Leach et al, 1993) occurs rarely in ovarian cancer (Foulkes et al, 1995). Mutations of p21 are infrequent in ovarian tumours, but varying levels of p21 expression were observed which did not correlate with p53 expression (Milner et al, 1996). However, there is evidence that p21 is associated with better differentiation status and tumour grade, and in this series, p21 positive staining was associated strongly with good survival of patients with ovarian cancer when tumours with mutant p53 were excluded (Al-Azraqi et al, 1996).

#### *1.4.3.3 Mutation of Oncogenes and Signal Transduction Pathways in Ovarian Cancer*

Much of the interest in dominantly acting genes in ovarian tumours has centred on the role of growth factors and their receptors. Epidermal

growth factor receptor (EGFR, p170), erbB-2 (HER-2 / neu, p185) and macrophage colony stimulating factor receptor (FMS, p165) are all trans-membrane proteins with tyrosine kinase activity in their cytoplasmic domains and over-expression of each has been shown to correlate with poor prognosis in epithelial ovarian cancer. The effect of ligand binding is to activate the tyrosine kinase domain, which in turn phosphorylates tyrosine residues on other cytoplasmic enzymes such as phospholipase C-gamma which cleaves diacylglycerol from membrane phospholipids, generating a second messenger with powerful mitogenic activity (Bast et al, 1993; Berchuck et al, 1994).

Twenty percent of ovarian cancers exhibit HER-2 / neu gene amplification, though over-expression at the protein level is demonstrable by immunohistochemistry in a somewhat higher proportion (around 30%) (Berchuck et al, 1990; Slamon et al, 1989). Immunohistochemistry is only a semi-quantitative technique and its interpretation can be subjective. Nevertheless it carries the advantage that tumour cells can be assessed on an individual basis without the problems associated with stromal and / or admixed normal epithelial cells encountered in tissue extracts.

Production of anti-p185 (HER-2 / neu) antibody has potentially profound therapeutic implications since it inhibits the growth of erbB-2 expressing tumours. This effect has been shown to involve down-regulation of intracellular diacylglycerol production (Deshane et al, 1994).

In over 80% of epithelial ovarian cancers, cultures established directly from fresh biopsies and examined within 24 hours revealed double minute chromsomes in at least a proportion of the cells (McGill et al, 1993). Double minutes are unstable genetic elements carrying amplified portions of the genome that commonly include dominantly activating oncogenes. Their presence at such high frequency in ovarian cancer highlights the importance of oncogenes in this disease. In some instances, the growth factor receptor genes mentioned above are likely

to be incorporated in the double minutes but other oncogenes are also implicated in ovarian cancer. This double minute DNA has been microdissected, PCR amplified and fluorescently labelled as a FISH probe. The unique amplified sequence hybridised with 8q24, the site of c-myc (McGill et al, 1996).

c-myc amplification and / or over-expression has been detected in over 20% of ovarian cancers, particularly of serous histology (Yokota et al, 1986). Where it is amplified or over-expressed, it may present a therapeutic target since, for example, cyclopentone antitumour prostaglandins inhibit c-myc expression, causing a G1 block and leading to apoptosis of tumour cells (Kikuchi et al, 1994)

Amplification of the ras gene family does occur (Filmus et al, 1985) but more commonly they appear to be activated by point mutations. In some studies, mutation of K-ras was noted, more often in borderline than in malignant ovarian tumours activity (Bast et al, 1993; Berchuck et al, 1994); but other reports suggest that K-ras mutations are usually associated with advanced stage cancers (Chien et al, 1993; Park et al, 1995)

A number of growth factors with possible autocrine functions have been investigated in ovarian cancer. The fibroblast growth factor genes fgf3 and fgf4 are among these and amplification of fgf4 in particular appears to correlate with advanced FIGO stage (Jaakkola et al, 1993; Rosen et al, 1993).

The macrophage colony stimulating factor (CSF-1) is the natural ligand for p165 c-fms and over-expression of either is associated with poor prognosis (Baiocchi et al, 1991). CSF-1 not only stimulates proliferation of ovarian cancer cell lines but also stimulates invasive behaviour in vitro, an effect which is probably mediated through urokinase (Chambers et al, 1995). It is known that the expression of urokinase plasminogen activator correlates strongly with tumour invasiveness in vivo.

### 1.4.4 Ovarian Cancer Progression: Attachment, Invasion, Angiogenesis and Metastasis.

#### *1.4.4.1 Tumour cell attachment, migration and invasion*

The capacity of neoplastic cells to invade and metastasize are fundamental distinguishing features of benign from malignant tumours.

The basement membrane, a thin continuous sheet which separates epithelial cells from adjacent stroma, plays a critical role as a barrier against invasion. The predominant components of the basement membrane are laminin, a large multifunctional glycoprotein, collagen IV and heparan sulphate proteoglycan. Basement membranes form barriers that block the passage of cells and macromolecules but become permeable at sites of tissue development, repair, inflammation and where the membrane contacts invasive malignant neoplasms.

The complex process of invasion consists of three principal steps whereby an invasive malignant neoplasm crosses its tissue compartment boundary to access and grow within an inappropriate tissue site, initially in adjacent stroma, and later, as a metastasis at a distant site. Tumour invasion is a process that consists of three major steps: a) tumour cell detachment/attachment; b) enzymatic degradation of the basement membrane; and c) migration of tumour cells to the adjacent tissue compartment.

a) Tumour cell detachment and adhesion

In order to invade, a tumour cell must firstly detach from its neighbour and then attach to the underlying basement membrane. Cadherins, cell adhesion molecules that mediate intercellular attachment, maintain attachment between epithelial cells. Down regulation or loss of cadherin expression is associated with invasiveness in carcinomas.

An optimal level of adhesion (attachment to the basement membrane) is required for cancer cells to successfully invade. Adhesion of cells to the basement membrane is mediated by a family of cell surface receptors called integrins. Integrins are heterodimeric protein

complexes which link cytoskeletal microfilaments (e.g. Actin, vinculin and talin) to extracellular matrix (ECM) proteins such as laminin, fibronectin, collagen and vitronetin. Integrins mediate their binding through a specific tripeptide motif, and this tripeptide has been shown to inhibit tumour cell invasion in-vitro and in-vivo. A balance of integrin expression is thought to be required for optimal invasiveness of tumour cells. Integrin under-expression does not allow tumour cells to adequately attach to the basement membrane (reviewed in Aznavoorian et al, 1993) Similarly, integrin over-expression may inhibit the capacity of tumour-cells to dis-attach from the basement membrane and therefore render those cells less invasive (Giancotti and Ruoslahti, 1990). Over-expression of a specific 67 kD laminin receptor (67LR) was first described by Lance Liotta and shown to be important for cancer cell adhesion to and invasion into the extra-cellular matrix (Liotta et al, 1984) and over-expression of 67LR has been associated with progression of various cancers (Pellegrini et al, 1995). Expression of 67LR has been shown to be increased in patients with progressive ovarian cancer (van den Brule et al, 1996). OVCAR3 cells incubated with laminin peptide fragments prior to intra-peritoneal injection failed to induce tumours in nude mice (Olt et al, 1996).

b) Basement Membrane degradation by tumour cells

Tumour cells generate a zone of lysis at the site of their attachment of the basement membrane. This is achieved by production of proteolytic enzymes which are secreted as pro-enzymes and have both activators and inhibitors to regulate their activity.

Several families of proteases are produced, the most important of which are the matrix metalloproteinases (MMPs). This family can be grouped into 3 types according to their substrate specificity: MMP-1 or interstitial collagenase (Templeton et al, 1990) degrades fibrillar collagens (types I, II and III). MMP-2 and MMP-9 are the type IV collagenases (Collier et al, 1988). MMP-3 or stromelysin consists of 3 types (Wilhelm et al, 1987). Stromelysin-1 (which degrades laminin)

stromelysin-2 (degrades fibronectin) and matrilysin (degrades proteoglycan core proteins). A cluster of such matrix metalloproteinases resides on chromosome 11q. The transcriptional regulatory regions of MMP genes contain binding sites for the transcription factors Ets and AP-1. In transfection assays, these transcription factors induce the expression of matrix metalloproteinase RNAs and thereby confer the invasive phenotype of MCF-7 breast cancer cells (Kaya et al, 1996).

Three other classes of protease are involved in this proteolytic activity, the most important of which is the serine protease uPA (urokinase-type plasminogen activator) whose expression appears to be closely related to enhanced invasion and metastasis. It converts inactive plasminogen to the active enzyme plasmin which can degrade collagen, fibronectin, fibrin and laminin (Liotta et al, 1981)

The expression of these proteolytic activities is tightly regulated. Natural Tissue inhibitors of metalloproteinases (TIMPs) are present to regulate the activity of MMPs, and localised secretion of MMPs until the effect of ubiquitously located TIMPs is locally overcome provides a highly focal degradative process which has a normal role in repair, tissue remodelling and the response to infection (Khokha et al, 1989).

c) Tumour cell migration

Having degraded the basement membrane the tumour cell must actively migrate to the adjacent tissue compartment that it will occupy. Cell motility, a normal cellular function, begins with polarisation and orientation of the cell followed by formation of pseudopodia (by membrane extension) at the cell's leading edge. The pseudopodium then forms attachments with the ECM and produces a contractile force to move the cell forward by a series of attachment and dis-attachment steps involving the cellular receptors that bind the matrix. Specific signals give direction and urgency to migration, including matrix components, growth factors, and scatter factors. Tumour cells additionally produce autocrine chemotactic factors.

The biology of ovarian cancer and the function of genes in other tumours suggests many possible candidates of which the above are examples but by no means an exhaustive list. Few of these genes have been shown to have an important role in ovarian cancer as yet. The human genome mapping project will undoubtedly produce many more candidates worthy of study, some as part of the project, but others by positional cloning of ovarian cancer associated genomic regions. The aim of this thesis was to take this approach in the light of genetic studies of loss of heterozygosity taking into consideration the clinicopathological features which distinguish ovarian cancer

## 1.5 The Role of Chromosome 11 in Ovarian and Other Neoplasms

### 1.5.1 Loss of heterozygosity of Chromosome 11p

LOH studies in many tumour types have provided evidence for putative tumour suppressor genes located on chromosome 11.

Table 1.3 outlines the major studies that have defined regions of LOH on the short arm of solid tumours other than ovarian cancer.

**Table 1.3 Studies demonstrating chromosome 11p LOH in cancers other than ovarian**

| Tumour | Region of LOH | Reference |
|---|---|---|
| Wilms' tumour | 11p13 | Fearon et al 1984, Orkin et al 1984, Reeve et al 1984 |
| Hepatoblastoma | 11p | Koufos et al 1985 |
| Sporadic Wilms' tumour | 11p15.5 | Reeve et al 1989 |
| Bladder cancer | 11p15.5 | Fearon et al 1985 |
| Rhabdomyosarcoma | 11p15.5 | Scrable et al 1987 |
| Lung carcinoma | 11p15.5 and 11p13-q13 | Weston et al 1989 |
| Breast carcinoma | 11p15.5 | Ali et al 1987 |
| Testicular teratoma | 11p15.5 | Lothe et al 1989 |

Two regions, 11p15.5 and 11p13 (site of the Wilms' and WAGR loci) repeatedly appear as important loci in these tumour series. It is important to note that most of these studies were performed in the 1980's, prior to PCR and access to abundant, highly polymorphic, well localised markers.

In ovarian cancer, frequent LOH has been demonstrated at 11p15 (Lee et al, 1989; Lee et al, 1990; Kiechle-Schwarz et al, 1993; Gallion et al, 1992; Vandamme et al, 1992; Viel et al, 1992; Eccles et al, 1992) although not all studies have confirmed this high level of loss (Zheng et al, 1991; Sato et al, 1991; Yang-Feng et al, 1992). A proximal locus at 11p13 (site of WT1) exhibits lower levels of LOH in ovarian cancer (Vandamme et al, 1992; Viel et al, 1992; Bruening et al, 1993). A minority of these studies have proposed a correlation of 11p LOH with poorly differentiated (Zheng et al, 1991; Kiechle-Schwarz et al, 1993) and more advanced (Viel et al, 1992) tumours, but in general patient numbers have been small and follow-up times short. Table 1.4 summarises the literature for chromosome 11p LOH in ovarian cancer (12 publications)

**Table 1.4 Ovarian cancer LOH rates on chromosome 11p**

| Loci | Position | LOH/HET | % LOH |
|---|---|---|---|
| Ha-ras | 11p15.5 (dist.) | 66/175 | 38% |
| INS/HB | 11p15.5 (prox.) | 51/169 | 30% |
| CALC | 11p15.4 | 17/46 | 37% |
| PTH | 11p15.2 | 9/29 | 31% |
| S17/FSH/S16 /CAT | 11p13 | 27/94 | 29% |

Three candidate tumour-suppressor genes are found on chromosome 11p. p57 is a cell cycle gene located at 11p15.5. This has been excluded as the gene responsible for tumour suppression in Wilms' tumour cell line suppressed hybrids (see below) and appears not to be involved in sarcomas which commonly have disruption at 11p15.5. WT1 has been investigated in ovarian cancer. Despite 39% LOH in this region, Bruening and colleagues showed that WT1 was not mutated in ovarian cancer (Bruening et al, 1993).

The third tumour suppressor, KAI-1 (Dong et al, 1995, see below), a prostate cancer metastasis suppressor, has not been investigated in ovarian cancer.

### 1.5.2 Loss of heterozygosity of Chromosome 11q

**Proximal chromosome 11q LOH**

Analysis of chromosome 11q has highlighted a different pattern of solid tumour involvement.

Proximally, allele imbalance at 11q13 has been reported for cervical cancer (Srivatsan et al, 1991a), neuroblastoma (Srivatsan et al, 1991b) multiple endocrine neoplasia type1 (Fujimori et al, 1992; Larsson et al, 1988), melanoma (Tomlinson et al, 1993), and B cell tumours (Williams et al, 1993)

Molecular studies of the proximal 11q region have shown low levels of allele imbalance in ovarian cancer (Foulkes et al, 1993; Li et al, 1991; Lee et al, 1989; Sato et al, 1991; Viel et al, 1992).

**Distal chromosome 11q LOH**

Early molecular studies showed abnormalities in human leukaemias at 11q23 (Zieman-van der Poel et al, 1991), RCK(lymphoma) cell line at 11q23 (Akao et al, 1992), hereditary paragangliomas at 11q23-qter (Heutnik et al, 1992) and Ewing's sarcoma at 11q24.

Until 1994, the only study that looked specifically at distal 11q in common solid tumours was in fact performed by Foulkes and colleagues studying ovarian cancer (Foulkes et al, 1993). They recorded a high level of LOH at 11q23.3-qter in a small sample of tumours. Following this report the telomeric half of 11q has been the subject of extensive investigation in many studies of solid tumours (see Table 1.5).

**Table 1.5 Studies demonstrating 11q LOH in cancers other than ovarian**

| Tumour | Regions involved | LOH rate | References |
|---|---|---|---|
| Colorectal carcinoma | Telomeric to 11q22.1 | 59% | Keldysh et al 1993 |
| Cervical carcinoma | 11q22-q24 | 44% | Hampton et al 1994 |
| Breast carcinoma | 11q23-q24, two regions | 45% | Negrini et al 1995 |
| Lung adenocarcinoma | 11q22-q24, three regions | 63% | Rasio et al 1995 |
| Malignant melanoma | 11q23 | 67% | Tomlinson et al 1993 |

The salient features of these investigations are the definition of several regions of LOH within the interval 11q22-q25 in several solid tumour types. Many of these regions were defined concurrent with or post-dating my studies, I will present and discuss these later.

Foulkes and others did demonstrate that 11q LOH was infrequent centromerically but much higher telomerically, and this is outlined in Table 1.6.

**Table 1.6 Ovarian cancer LOH rates on chromosome 11q**

| Loci | Position | LOH/HET | % LOH |
|---|---|---|---|
| CD20/S146 /FGF3/FOLR1/ TYR | 11q12-q14 | 12/104 | 12% |
| S85/ STMY-1 /S144/S29 /CD3D/S147 | 11q22-q23.3 | 29/73 | 40% |
| S147 | 11q24.1 | 8/12 | 67% |

The gene for Ataxia-Telangiectasia (ATM) was recently identified and is located at 11q22-q23 (Savitsky et al, 1995) It is postulated that ATM heterozygotes may be at increased risk of breast cancer but recent mutation analysis revealed that there were no significant ATM mutations in a cohort of patients with breast cancer (Vorechovsky et al, 1996). Analysis has not yet been performed in ovarian cancer. The progesterone receptor is also a candidate tumour-suppressor gene. The preceding discussion on hormonal factors (see above) has outlined the inhibitory role of PGR on breast and ovarian cancer growth. A constitutional polymorphism in intron G of the PGR gene has been shown to be associated with an increased incidence of sporadic ovarian cancer, suggesting a potential role for PGR as a tumour suppressor (Rowe et al, 1995).

## 1.6 Somatic cell genetics and the functional identification of tumour-suppressor gene regions

### 1.6 Somatic Cell Hybrids

#### 1.6.1 Historical introduction

The development of somatic cell hybrids in 1960 (Barski et al, 1961), which are cells containing all or a part of the genetic complement of two distinct somatic cell lines, has proven to be of immense importance in the study of mammalian genes. Although it was clear from the outset that these resources would be useful, it was not until methods became available for enriching for the hybrids above background non-hybrid cells that their potential was unleashed for gene mapping and complementation.

Selectable systems

In 1964, Littlefield fused together two mouse cell lines each containing different negative selectable markers. One cell line was deficient in hypoxanthine-guanidine phosphoribosyl transferase (HGPRT), the other was deficient in thymidine kinase (TK). Each of these enzymes are required to synthesise the appropriate nucleotides from the nucleosides hypoxanthine and thymidine respectively when the de-novo nucleotide synthetic pathway is blocked by aminopterin (an inhibitor of dihydrofolate reductase, DHFR). Therefore, in the absence of HGPRT or TK the cell will not grow on a medium containing hypoxanthine, aminopterin and thymidine (HAT). Littlefield's cell fusion experiment allowed the HGPRT and TK from each cell containing that enzyme to cross-complement the $HGPRT^-$ and $TK^-$ phenoypes thereby allowing only the somatic cell hybrid to grow in HAT medium (Littlefield, 1964). This enrichment or selectable system, which remains an important tool today, provided the impetus to devise other selectable systems for use in somatic cell genetics culminating in the development of a series of bacterial dominant

selectable markers utilised for selection of tagged eukaryotic chromosomes and cells (see below).

### 1.6.2 Inter-specific somatic cell hybrids

The first inter-specific somatic cell hybrids (produced by fusion of cells from different species) were produced between mouse and human cells by Harris and Watkins in 1965 (Harris and Watkins, 1965). The creation of inter-specific hybrids allowed the human chromosomal complement to be segregated into one or a few chromosomes in a rodent chromosomal background, and this allowed the characterisation of one or a few chromosomes at a time. The result was an explosion in the number of cloned genes and markers mapped cytogenetically.

Reduced inter-specific hybrids which complemented the recessive genetic defect of a $TK^-$ mutant rodent line demonstrated the second great opportunity of somatic cell hybridisation: the localisation of the gene complementing the mutant phenotype to a chromosome, thereby allowing workers a glimpse of our ability in the future to clone eukaryotic genes by their function (Migeon and Miller, 1968). The development of Giemsa banding (Sumner et al 1971), immuno-chemical identification of cell surface antigens (reviewed by Tunnacliffe et al 1983), and the advent of nucleic acid hybridisation technology (Southern, 1975) allowed strategies for rapid characterisation of somatic cell hybrids, establishing them as powerful tools for biology.

### 1.6.3 Somatic cell hybrids, gene mapping and positional cloning

As indicated above, the ability to unambiguously demonstrate physical linkage of a gene to a particular chromosome became a reality with the development of monochromosome inter-speciic hybrid panels. These panels have been produced from disparate sources. Some were produced by fortuitous reduction segregation events that remained

stable despite lack of a selectable marker, such as chromosome 11 in CHO cells (Kao et al 1976). Other hybrids were generated using natural cross complementation of an interspecific mutation such as $TK^-$ mouse lines and human chromosome 17 (Migeon and Miller 1968). However, the most robust method has proven to be the introduction of dominant bacterial selectable markers into human chromsome prior to the creation of the hybrids (Koi et al, 1989a).

Having assigned a cloned gene or sequence to a chromosome, the next step is to localise it precisely either for mapping purposes (in the case of a cloned gene) or for positional cloning (in the case of a sequence physically closely linked to an un-cloned gene). Fragmentation of chromosomes in hybrids allows panels to be generated containing stable sub-chromosomal segments providing a useful way of assigning a locus to a particular region of chromosome. Chromosomal rearrangements such as translocations or deletions may either occur spontaneously or be induced in culture and mapping loci with respect to the rearrangement helps in regional assignment. The ability of gamma irradiation to cause gross chromosomal damage has been used to generate fragmented and rearranged chromosomes for mapping. Important experiments by Goss and Harris showed that irradiating human lymphocytes before fusing them with $HPRT^-$ rodent cells generated somatic cell hybrids with fragmented chromosomes. Growth on HAT medium identified rearranged fragments of the X chromosome clustered around the human HPRT locus. Furthermore they were able to establish an ordered set of physically linked genes in the vicinity of this locus (Goss and Harris, 1975). The irradiation fusion gene transfer (IFGT) strategy has been scaled up and combined with two powerful techniques; the polymerase chain reaction (PCR) and fluorescence in-situ hybridisation (FISH) to provide detailed physical maps of whole chromosomes. A particularly good example has been the construction of a detailed sequence tagged site (STS) map incorporating integrated information from microsatellites, cloned

genes, and expressed sequence tags (ESTs) spanning the whole of human chromosome 11 (James et al, 1994). Using the J1 CHO somatic cell hybrid which contains human chromosome 11 in a Chinese hamster ovary background (Kao et al 1976), Richard III and colleagues generated a panel of 102 human-hamster radiation hybrid (RH) cell lines by X-irradiation of the J1 cells (Richard III et al, 1991; Richard III et al, 1993). From these cell lines, James and colleagues selected 86 RH cell lines which formed overlapping fragments spanning the whole of human chromosome 11. The analysis of sufficient numbers of hybrids ensured that any conflicting data produced by intra-chromosomal rearrangement due to radiation fragmentation is statistically neutralised. Distances between adjacent markers were estimated in $cR_{9000}$ (cR). A distance of 1 cR (centi ray) between two markers corresponds to a 1% frequency of breakage between the markers after exposure to a 9000 rad dose of X-rays. The total length of the chromosome 11 RH map was found to be 2867 cR. Based on an estimated size of 144 Mb for the chromosome (Morton 1991[PNAS]), James et al suggested that 1 $cR_{9000}$ was equivalent to 50.2 Kb (James et al, 1994). This figure is consistent with estimates from other chromosomes (Cox et al, 1990; Abel et al, 1993). By analysing the patterns of co-segregation of markers by PCR (whether each STS is present or absent in each hybrid), 506 STSs were mapped into 299 unique positions on the chromosome with odds of >10:1. The average resolution between markers was high at 480 Kb. A more robust subset of markers ordered with odds of at least 1000:1 formed an underlying framework of this map consisting of 260 STSs at 143 unique positions giving an average resolution of the map at 1 Mb. This integrated, highly ordered physical map is clearly the way forward towards construction of robust STS based physical maps using YAC contigs. The provision of a physical RH STS map provides an attractive reference for building and cross-screening of YAC contigs in order to avoid the problems caused by chimaerism. As a starting point for the mapping

component in this thesis, I used this radiation hybrid map as a way of ordering LOH data from patient specimens and relating it to physical mapping data from microcell fusion experiments.

### 1.6.4 Somatic cell hybrids and functional gene cloning

Somatic cell genetics from the outset held an alluring promise of cloning genes by function. Complementation of a readily isolated phenotype, e.g. a cell surface marker, allows a rational approach to heirarchical strategies whereby whole cell fusion, then microcell mediated chromosome transfer can delineate the chromosome and perhaps the chromosomal segment on which the gene resides. Unfortunately, there is no direct route to the gene beyond this, although complementary strategies can be brought to bear on the problem. One can a) transfer stable subchromosomal fragments, b) prepare DNA for transfection either from mitotic plate preparations or by standard preparation of naked DNA, c) adopt a difference cloning approach to clone cDNAs, or d) take a laborious positional cloning approach (as outlined above).

Cell lines with stable subchromosomal fragments generated by X-irradiation of packaged chromosomes prior to their transfer to other cell lines (XMMCT, see microcell mediated chromosome transfer below) can be used for complementation to refine the position of a gene of interest (Dowdy et al, 1990; see below). Microcells prepared from the somatic cell hybrid can be irradiated prior to microcell fusion and transferred back to the rodent cell line to create somatic cell hybrids with fragmented human chromosomes that have not re-arranged with rodent chromosomes. The use of homologous recombination to multiply target the human repetitive component of monochromosome somatic cell hybrids with a dominant selectable marker has been successfully achieved (Watson et al, 1995). Introduced with the dominant selectable marker are a variety of useful features, including unique restriction sites and markers to allow rescue of the human DNA fragments into yeast and bacterial vectors. X-MMCT

using these hybrids can generate a variety of non-rearranged stable subchromosomal somatic cell hybrids carrying a dominant selectable marker. The use of these fragmented donor cell lines to complement the desired phenotype by MMCT in a biologically appropriate cell line and assay creates the next resource for the heirarchical approach to functional cloning: a cell line which is functionally complemented bearing a targeted cloning vector nearby. If the assay can be repeated in cell lines from a different species, cloning of regions of the transgenome is more readily achievable. However, many clinically relevant phenotypes are human and tissue specific (e.g., tissue restrictions of tumour suppressor gene effects in specific tumour types) and therefore intra-specific functional assays may be more relevant.

Increasingly, transgenic workers have been able to perform stable YAC transfer into ES cells to study the expression of stretches of chromatin containing genes of interest which have been manipulated (Van Heyningen et al, 1995b; Schedl et al, 1996). These approaches have not however met with success in somatic cells, although as a technical excercise, they should ultimately yield to refinement of the technique.

The next step Heirarchical step down in practice currently is to perform chromosome mediated gene transfer (McBride and Ozer 1970, Klobutcher and Ruddle 1979), whereby calcium phosphate transfection of isolated mitotic chromosomes is performed on the same recipient cells used for MMCT, and the same assay is again selected for along with the dominant selectable marker. This time the selectable marker with cloning vector and 1-50 Mbp of flanking DNA are transferred with the gene responsible for the observed functional complementation. Analysis of this transgenome and its subsequent direct cloning by preparative pulse field gel electrophoresis (PFGE) into yeast or bacterial chromosome vectors allows further characterisation of the functional region in order to clone the gene of interest.

The main drawbacks of CMGT are firstly, co-transfer of discontinuous non-selected DNA since unlike MMCT there is little regulation over

the amount of chromatin transferred and its rearrangement. Secondly, re-arrangements sustained by transferred chromatin between the selectable marker and the functional region may be misleading. Thirdly it is preferable to use a complemented cell line as a source for CMGT since performing CMGT directly from the inter-specific donor somatic cell hybrid runs the risk of complementing the defect with discontinuous co-transferred conserved murine genes of similar function.

Two interesting PCR based techniques have been developed to clone expressed sequence differences between functionally complemented hybrids. The first technique, differential display, uses random PCR primers to identify expression differences by RT-PCR which can then be cloned and lead to identification of transferred or differentially regulated expressed sequences. The second (and much more powerful) method is an adaptation to study cDNA populations of representational difference analysis (RDA, Lisitsyn et al, 1993). cDNA-RDA consists of subtraction hybridisation between restricted cDNA populations from the non-complemented and complemented cell lines. The population of cDNAs expressed by the complemented phenotypes are then amplified, and the process is repeated twice more until all co-expressed sequences are eliminated leaving only sequences specifically expressed by the complemented phenotype (Hubank and Schatz, 1994). A potential problem is the expression of a point mutated transcript in the non-complemented cell line, however, this is clearly a powerful complementary approach to the strategies discussed above.

### 1.6.5 Suppression of the cancer phenotype by somatic cell hybridisation

Barski (Barski et al, 1961) had shown that when highly malignant mouse cells were grown in a mixed culture with mouse cells of low malignancy, spontaneous hybrids arose one of which remained highly malignant. Fusion of this highly malignant line with mouse fibroblasts also produced a hybrid line which was highly

malignant. These studies suggested that malignancy behaved as a dominant phenotype. However, these hybrids had not been sufficiently analysed karyotypically; chromosome losses could have occurred in the hybrids and have been selected for by increased growth rate. Harris and co-workers re-investigated the phenotype of hybridisation of malignant with non-malignant cells and published their results in 1969 (Harris et al 1969). They used three murine malignant cell lines, one a murine mammary carcinoma (Ehrlich tumour) and two murine sarcomas (SEWA, an osteogenic sarcoma, and MSWBS, a myosarcoma) all of which had been converted to a lethal ascites model. These malignant cells were fused using sendai virus with mouse A9 fibroblast cell line, a non-malignant cell line. Harris demonstrated that all three malignant cell lines were suppressed for malignancy when fused with A9 cells. They also showed that these hybrids produced revertants back to the malignant state in association with loss of chromosomes. This study suggested that the neoplastic phenotype, far from being a dominant trait was in fact a recessive phenotype, complemented by restoration of 'normal' genetic material.

Despite these elegant findings, many other workers found these results difficult to repeat. It became obvious that loss of chromosomes from the cell hybrids due to karyotypic instability was responsible of re-expression of tumorigenicity. These revertants were particularly obvious in rodent intraspecific hybrids (formed by fusion of rodent tumour cell lines with rodent "normal" cell lines) and human/rodent interspecific hybrids. Stable tumour-suppressed hybrids were finally generated utilising human intraspecific fusion (Stanbridge, 1976). Revertants to tumorigenicity occurred fairly infrequently in these hybrids. Careful karyotypic analysis of these tumorigenic revertants did in some cases allow workers to make the association of tumorigenic reversion with the loss of a chromosome. Although this does not constitute proof, it is a powerful piece of circumstantial evidence. The

most persuasive such piece of evidence came from studies utilising intraspecific HeLa X human fibroblast cell hybrids. Hybrids retaining both normal fibroblast chromosome 11 copies were completely suppressed for tumorigenicity. However, loss of one of the normal copies of chromosome 11 was associated with reversion to tumorigenicity (Stanbridge and Ceredig, 1981; Srivatsan et al, 1986).This suggested not only a specific tumour-suppressor element on chromosome 11 but a dosage effect whereby two genomic elements acting in a dose dependent manner were required to suppress tumorigenicity. It would have been extremely interesting to observe the effect on a tumorigenic revertant cell hybrid containing one copy of normal chromosome 11 of returning specifically a second normal copy of chromosome 11. However, at this time there was no specific method of returning a specific chromosome to a cell line and it required a technically difficult and prolonged period of method development before such an experiment became possible.

### 1.6.6 Microcell Mediated Chromosome Transfer

The observation of reversion to tumorigenicity of cell hybrids in association with specific chromosomal loss suggested an obvious and attractive experiment: to observe the functional effects of returning that chromosome to the hybrid. However, a convergence of cell biological technologies was required before such an experiment became a feasible, and the development of microcell mediated chromosome transfer (MMCT) allowed a chromosome to be transferred and stably retained as a discrete structural unit in succeeding generations of hybrid cells (unlike other methods of DNA transfer which result in rapid degradation of non-selected DNA). The retention of specific chromosomes would require their tagging with endogenous or exogenously introduced dominant selectable markers which could be used as a selectable system in the recipient cells.

In 1974, Ege and Ringertz were able to generate microcells; subnuclear particles containing a restricted subset of murine genetic material surrounded by a rim of cytoplasm and an intact plasma membrane. The incubation of murine cells with the mitotic inhibitor colcemid resulted in metaphase block, and continued exposure to colcemid resulted in a reformation of the nuclear membrane around the mitotic chromosomes scattered around the cell in the absence of a mitotic spindle producing the appearance of micronuclei. The cells were grown on plastic discs, which were placed in media filled centrifuge tubes and centrifuged at 12,000 x g for 20 minutes at $34^0$C in the presence of cytochalasin-B, an inhibitor of actin filament formation. This resulted in enucleation of >95% of the cells on the discs, and the resultant suspension contained the free microcells (Ege and Ringertz, 1974).

In 1977, Fournier and Ruddle were able to transfer murine chromosomes contained within microcells into mouse, human and Chinese hamster cell lines (Fournier and Ruddle, 1977). For the first time they were able to create viable somatic cell hybrids containing one, or a few transferred chromosomes. They used three mouse fibroblast cell lines: A9 which lacks both hypoxanthine phosphoribosyltransferase ($HPRT^-$) and adenine phosphoribosyl-transferase ($APRT^-$); B82, deficient in thymidine kinase ($TK^-$); and $CT11C_1$, an $APRT^-$, $HPRT^+$ line. Microcells were produced using the Ege and Ringertz method. The crude microcell preparation was purified by sedimentation on a bovine serum albumin gradient. The upper fractions were pooled, containing the smallest structures which included the purified microcells. Inactivated Sendai virus was used to attach and fuse the microcells to a monolayer of recipient cells. The cells were allowed to recover for 24 hours, split into a number of small flasks and selected on HAT medium (see above). Hybrid clones appeared after 2-4 weeks. Microcells from A9 cells were used to

complement the $TK^-$ phenotype of B82. The modal chromosome number of the intraspecific hybrids were consistent with transfer of one or a few chromosomes only. Interspecific hybrid were generated by transferring murine microcells into Chinese hamster and human (HeLa) cell lines.

The development of the technique of microcell mediated chromosome transfer (MMCT) was clearly a powerful development in somatic cell genetics, however micronucleation of normal diploid human cells proved to be extremely difficult (Tourian et al, 1978). Human cells typically produced micronuclei with only low efficiency, or the produced microcells were not viable.

In 1980, McNeill and Brown defined the conditions for micronucleation of human foreskin diploid fibroblasts; using high concentrations of colcemid they were able to micronucleate 40-60% of the cells (McNeill and Brown, 1980). Rather than BSA gradient purification, they filtered the crude microcell preparation through a 3 um Nucleopore polycarbonate membrane. The resultant preparation contained microcells, was free of whole cells and contained less than 1% contaminating karyoplasts. In contrast to the Sendai virus fusion protocol used by Fournier and Ruddle, McNeill and Brown used Phytohaemagglutinin-P (PHAP), a lectin, to attach the human microcells to the recipient mouse $TK^-$ cells and then used polyethylene glycol (PEG) to fuse the attached microcells with the recipient cells. The cells were selected on HAT medium and clones emerged at 2-3 weeks. Karyotypic analysis revealed that this technique reliably transferred a single human chromosome 17 into the murine recipient.

The ability to transfer a single human chromosome into a mouse cell line allowed the generation of interspecific human monochromosome somatic cell hybrids. These would have a central role in gene mapping and the elucidation of gene expression and regulation. McNeill and Brown's experiment had utilised a naturally sited selectable marker that could be utilised in their assay system. However, other

chromosomes did not have other such markers, and the next step in the creation of monochromosome somatic cell hybrids was the development of exogenously introduced selectable markers into human chromosomes prior to their transfer into mouse fibroblast cells.

More recently, a protocol has been developed that allows microcells to be generated directly from mitotic cells for MMCT (Stubblefield and Pershouse, 1992). This method used a short treatment of colcemid (2-5 hours) of proliferating donor cells to generate cells blocked in mitosis. The mitotic cells were shaken off the flasks and transferred to an isotonic percoll gradient buffered with HEPES and containing cytochalasin-B. After centrifugation at $37^0$C the microcell preparation was filtered, attached and fused in the usual way. The major advantages of this protocol were firstly speed and secondly that the majority of microcells contained only one chromosome since micronucleation of several chromosomes at once did not occur.

### 1.6.7 Introduction of dominant selectable markers into normal human chromosomes for MMCT.

The exogenous introduction of the XGPRT marker by transfection of the plasmid pSV2gpt into $HPRT^-$ HeLa cells, rendered them viable in HAT medium (Saxon et al, 1985). The randomly integrated plasmid allowed several chromosomes to be selected for after microcell fusion into an $HPRT^-$ mouse fibroblast cell line. Although these resources of human chromosome somatic cell hybrids were excellent for gene mapping, they could not be used widely for tumour-suppression studies, as the XGPRT marker was not dominant with respect to $HPRT^+$ human cells. However, the first examples of MMCT of human chromosomes from monochromosome somatic cell hybrids into human tumour cell lines were achieved using a t(X;11) carrying HPRT into HPRT deficient tumour cell lines (see below).

Generation of human chromosome somatic cell hybrids tagged with the truly dominant selectable marker neo, the E. coli neomycin resistance gene (which confers resistance to the antibiotic G418) was achieved in 1987 (Lugo et al, 1987). Human diploid fibroblasts were infected with a defective retrovirus derived from Moloney leukaemia virus and carrying the neo gene driven from its long terminal repeat promoter. The infected fibroblasts were grown in G418 and microcells were produced from them and transferred into the mouse fibroblast cell line 3T6 by MMCT. Somatic cell hybrids carrying dominantly tagged chromosomes 11, 14, 20 and 21, were generated which could be used for MMCT into any human cell line and selected for.

A larger series of neo-tagged human monochromosome somatic cell hybrids were subsequently generated by Koi and co-workers (Koi et al, 1989a, see above). They transfected normal human fibroblasts (MRC-5 and NT1-4) with pSV2-neo plasmid DNA and isolated G418 resistant clones. From these they performed MMCT into mouse A9 cells, and isolated 13 stable microcell hybrid clones containing a dominantly tagged human monochromosome 1, 2, 5, 6, 7, 8, 10, 11, 12, 15, 18, 19 and 20. They confirmed these somatic cell hybrids as useable donors for MMCT into human recipient cell lines (Koi et al, 1989b).

More recently, a complete panel of 23 stable monochromosome somatic cell hybrids has been generated (Cuthbert et al, 1995). The human chromosomes were tagged with a selectable fusion gene Hytk and introduced in a retroviral vector into normal human fibroblasts, and then transferred into mouse A9 cells. Hytk allows both positive (Hygromycin) and negative (Ganciclovir) selection. The latter marker allows for "flip-flop" demonstration of phenotype reversion, useful in confirmation of chromosome associated functions.

A more sophisticated approach to tagging has been adopted by Watson and co-workers (Watson et al, 1995). This involved the introduction of a dominantly selectable marker specifically into the human component of the somatic cell hybrid. Watson constructed vectors

containing the neo-marker and human-specific repeat sequences. Vectors containing the Long interspersed nuclear element (LINE) L1 repeats did specifically mediate insertion into the human chromosome by homologous recombination in J1-C4, a human monochromosome 11 somatic cell hybrid in Chinese hamster ovary background. Other unique features of the vector were the presence of the yeast selectable marker Leu2 for cloning the fragments of chromosome DNA in yeast, and the bacterial selectable marker Amp for cloning in cosmids. Additionally, the unique restriction sites I-SceI and LacO provided unique cutting sites for the integrated vector. This resource provides a direct route from functional effect by whole chromosome introduction to cloned fragments in yeast or bacterial vectors. Repeat mediated targeting can generate human chromosomes tagged at many sites, providing an obvious route to reduced or fragmented hybrids.

### 1.6.8 Cell Biological assays for the cancer phenotype

#### *1.6.8.1 In-vivo studies of the cancer phenotype*

Conventional, but simplistic, definitions of the function of tumour suppressor genes have led to arbitrary implementation of a gold standard. In his influential review, Stanbridge states that "there is no facile method for assaying tumour suppressor function other than the in-vivo tumorigenicity testing in immune deficient mice". Using the narrow definition that a tumour suppressor gene is a gene that completely stops the development of a tumour, then Stanbridge is correct, since no assay provides an assessment of in-vivo tumorigenicity as satisfactorily as an assay of in-vivo tumorigenicity!

However, if one examines Stanbridge's own definition of a tumour suppressor gene, he states "[the phenomenon of] tumour suppression indicated that a gene (or genes) from a normal cell might replace a defective function in the cancer cell and render it responsive to normal regulators of cell growth". This is, of course, intuitively much more satisfactory as a definition, and immediately suggests that the

above gold standard is not a gold standard at all: suppression of tumorigenicity is clearly a highly specific assay: when suppression occurs one has clearly introduced a tumour suppressor gene. However, this assay is not very sensitive, and failure to suppress tumorigenicity in nude mice cannot be taken to mean that the gene of interest is not a tumour suppressor gene (by Stanbridge's own definition above).

The ability of malignant tumours to spread from their original site to form metastases is the main obstacle to successful cancer treatment. The complexities of the metastatic process involve not just tumour cells, but also host cells and the extracellular matrix. The process begins with tumour cells disattaching from their normal site, attaching to the basement membrane, penetrating the basement membrane, invading the underlying stroma by migration, local destruction and proliferation. Accessing blood vessels by an active angiogenic or invasive process, or accessing lymphatics allows tumour cells to disseminate to distant sites. At those sites, specific processes of attachment, local destruction and proliferation occur, followed by further spread.

Given its complexities, there may be components of the cancer process that can not be detected using the subcutaneous tumorigenicity assay.

Other in-vivo assays are increasingly used to assay for tumour suppressor genes.

One type of assay begins with the premise that solid tumours rarely metastasise under the skin. Attempts have therefore been made to implant cancer cells orthotopically to the site of origin of the tumour and observe its behaviour from the primary site, e.g. inoculation of ovarian cancer cells under the ovarian surface epithelium of nude or SCID mice. This is a demanding technique and although it may have the shortcoming that the cancer cell lines may have been developed from metastases in the first place, it is a more rational approach to assessing contextual behaviour of cancer cells hopefully in an appropriate autocrine and paracrine milieu, and one can observe the

entire cancer process as it might happen naturally after establishment of a primary tumour (Fu et al, 1993).

Another type of assay addresses the possibility that although a suppressor gene may not inhibit primary tumour formation, its inactivation is an essential step for cancer cells to metastasise from their primary site. Injection of cells into the bloodstream followed by counting either pulmonary or splenic metastases after a suitable time interval have been developed successfully and several chromosomes introduced into particularly melanoma, prostate and breast cancer lines have demonstrated suppression of metastasis forming ability with no perceptible influence on subcutaneous tumorigenicity (Dong et al, 1995; Miele et al, 1996; Phillips et al, 1996). More recently an assay for formation of intra-peritoneal metastases from ovarian cancer cells has been developed (Fu et al, 1993).

### *1.6.8.2 In-vitro assays of the cancer phenotype*

With increased understanding of the multiple components of the neoplastic process, somatic cell geneticists should turn their attention to more sophisticated in-vitro cell biological techniques that can assay specific components of this process.

Following initiation and promotion steps which alter the balance between cell proliferation and death, resulting in an immortal clonal tumour at a primary site, the first stage of the metastatic process involves interactions with the extra-cellular matrix, and can be separated into 3 defined sequential steps: attachment, proteolysis and migration. Thereafter angiogenesis not only ensures that the metabolic needs of the growing tumour are met, but also provides an obvious route for dissemination of tumour cells to other sites in the organism allowing establishment of metastatic deposits.

### *1.6.8.3 Tumour cell proliferation, death and the cell cycle*

Direct quantitation of cell growth on tissue culture plates allows basic functional effects on growth to be assessed. A property of cancer cell growth is that of serum independence. Thus progressive reduction in serum added to the culture media may accentuate the effect associated with introduction of a TSG. Effects on contact inhibition may be assessed by estimation of saturation density of the cells after prolonged continuous growth in-vitro. Surrogate endpoints such as cell doubling time may be estimated using these techniques.

DNA fluorescence activated cell sorting (DNA-FACS) analysis of propidium iodide stained cells allows comparison of the cell cycle distribution of the cells before and after introduction of a TSG to assess its involvement in cell cycle regulation (see cell cycle, above).

Several methods can be used to assess if perceived growth inhibition may be due to the introduction of senescence or pro-apoptotic functions. Direct observation of growing cells and of material shed into tissue culture media is a simple but valuable methods of assessing growth arrest and cell death. FACS analysis can indicate early changes in apoptosis (Annexin V assay) and combined early and late markers of cell death (Fluoro-TUNEL test).

The clonogenic soft agar assay

The capacity of tumour cells to grow on a non-solid surface is a defining characteristic. Several assays have been developed based around the ability of tumour cells to grow in soft (0.3%) agar. The clonogenic soft agar assay correlates most closely as an in-vitro test with in-vivo tumorigenicity.

### *1.6.8.4 Invasion assays*

Connective tissues have been used to assess tumour cell invasion in-vitro. However, tissue systems such as bladder wall (Hart and Fidler, 1978), amnion (Liotta et al, 1980), chicken chorioallantoic membrane (Marcel et al, 1981), and lens capsule (Starkey, 1984) have proved

difficult to standardise. In order to improve reproducibility and introduce quantitation to the tumour cell invasion assay, reconstituted basement membrane has been used as the basis of several such assays. In 1986, Terranova and colleagues (Terranova et al, 1986) constructed a compressed disc composed of laminin and collagens type I and IV. They used this disc as a barrier to assess tumour cell invasion in a Boyden chamber (a modified tissue culture chamber composed of a fenestrated filter that allows the passage of cells upon which the disc is placed).

In 1982, Kleinman and colleagues had developed a method for extracting a multicomponent preparation of natural solubilised tissue basement membrane from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins (Kleinman et al, 1982). This preparation was commercially produced as a standardised preparation called Matrigel. Matrigel is composed principally of laminin and collagen type-IV. It also contains heparan sulphate proteoglycans, entactin and nidogen. It also contains TGF-B, FGF, tPA and other growth factors.

In 1987, Albini and co-workers used Matrigel to coat the porous filters of a Boyden chamber and thereby developed the first reproducible quantitative tumour cell invasion assay (Albini et al, 1987). Further refinements followed with the adaptation of the quantitative colorimetric MTT assay to the method in 1990 (Schlechte et al, 1990) and improvements in the uniformity of matrigel coating by water-repellent treatment of the Boyden chamber (Imamura et al, l1994).

### *1.6.8.5 Tumour cell attachment assay*

Attachment of cells to tissue culture plastic can be assessed quantitatively. Alterations in tumour cell attachment as a result of introduction of a TSG may be specific for receptors binding individual components of the ECM. The attachment assay therefore involves coating the tissue culture plastic with purified components of the ECM

such as collagen IV, fibronectin or laminin. The cells can then be assayed for their specific ability to attach to particular components of the ECM before and after introduction of a TSG, and therefore alterations in integrin pathway regulation as a result of TSG introduction can be addressed.

#### *1.6.8.6 Tumour cell migration assay*

In a similar way, introduction of a TSG may correct signal transduction pathways that allow the cell once more to respond to specific growth inhibitory signals, reflecting a reduction in cell migration. These assays are performed in transwells similar to Boyden chamber used in invasion assays.

### 1.6.9 Chromosome 11 tumour suppression studies in human cancer cell lines

The first demonstration of transfer of a single human chromosome to a human tumour cell line was reported by Saxon and colleagues in 1986 (Saxon et al 1986). A human fibroblast cell line was identified with a balanced translocation chromosome , t(X; 11) with 11pter-q23 translocated to Xq26-qter, thereby translocating the HPRT marker to chromosome 11. This cell line was fused to HPRT deficient A9 cells and a resulting somatic cell hybrid clone was used as a microcell donor line. A somatic cell hybrid between HeLa and normal human fibroblasts, originally non-tumorigenic, which had reverted to tumorigenicity and was also $HPRT^-$, was used as recipient.

Transfer of t(X; 11) to the hybrid and also to HeLa cell line suppressed subcutaneous tumorigenicity in nude mice whereas MMCT of the X chromosome did not. The HeLa tumour suppressor was localised to 11q by mapping of the microcell hybrids (Misra et al, 1989)

The same translocated chromosome 11 was shown to suppress tumorigenicity in the $HPRT^-$ variant of the Wilms' tumour cell line

G401. In the same series of experiments the X chromosome and a t(X; 13) did not suppress tumorigenicity of G401 (Weissman et al, 1987). Using a [der(11)t(X;11p)(q21;q13)] Dowdy and colleagues showed that 11pter-q13 suppressed the tumorigenicity of G401. They then generated two radiation reduced hybrids, one with a deletion of 11p15.5-p14.1 and the other with a deletion of 11p13-p12. Only the latter suppressed tumorigenicity. This excluded the WT1 locus (Call et al, 1990; Gessler et al, 1990)) as the tumour-suppressor gene in G401 Wilms' tumour cell line and suggested that the suppressor lay within the interval p15.5-p14.1. A second locus had been mapped in Wilms' tumour by LOH to 11p15.5 (Reeve et al, 1989), dubbed WT2, and Dowdy and colleagues therefore suggested that the functional locus mapped to this region (Dowdy et al, 1991). More recently, the CDK inhibitor $p57^{Kip2}$ was cloned by homology with p21 and p27 (Lee et al, 1995; Matsuoka et al, 1995) and was localised to 11p15.5, within a 500 kb region that includes WT2. Northern analysis comparing G401 parent line, G401/chr 11 suppressed microcell hybrids, and G401/chr 11 non-suppressed hybrids revealed no correlation of expression of the 0.8 Kb p57 transcript with suppression of tumorigenicity, suggesting that p57 is not WT2 (Reid et al, 1996).

In 1989, Koi and colleagues transferred a human chromosome 11 into SiHa, a human cervical carcinoma cell line. In 4 out of five clones, there was suppression of tumorigenicity, in the final clone there was a reversion event causing loss of the introduced chromosome and tumour forming ability in nude mice (Koi et al, 1989). This work was extended by the same group, demonstrating that chromosome 11 could suppress tumorigenicity of the human endometrial carcinoma cell line HHUA and the human rhabdomyosarcoma cell line A204 but that there was no effect of this chromosome in the human renal carcinoma cell line YCR-1 (Oshimura et al, 1990). The suppressor region was localised to 11p15 in rhabdomyosarcoma cells, but a second growth suppressor was mapped to the long arm in these cells since hybrids

carrying only the long arm were also inhibited for growth (Loh et al, 1992).

In an attempt to further localise the site of this 11p15 suppressor in rhabdomyosarcoma cells, Koi and colleagues constructed an elegant strategy using "subchromosomal transferable fragments" or STFs (Koi et al, 1993). Until this work, a direct route from functional whole chromosomes to a positional map of the region containing the suppressor gene was difficult to achieve. This is because the whole chromosome is a stable transferable fragment, the selectable marker is located distantly, and it is therefore too big to localise a gene region by any means other than a serendipitous rare breakage event located close to the gene. Although yeast artificial chromosome (YAC) transfer is a possible approach to localise the region, and has been achieved in somatic cells, thousands of specific YACs would be required to cover the chromosome. Furthermore, since the desired phenotype (growth suppression) is normally selected against, direct expression cloning of the suppressor gene of interest would be unlikely to be successful using this method.

Koi's elegant strategy consisted of taking a t(11;X) A9 somatic cell hybrid and transfecting it with a $neo^R$ plasmid. 18,000 $neo^R$ clones were pooled and used for MMCT to A9 cells, double selecting on HAT and G418. 90 clones that survived double selection were pooled and microcells were generated from them and gamma-irradiated prior to fusion. 85 $neo^R$ A9 clones were isolated and characterised. Nine 11p15 fragments were transferred to Chinese hamster ovary cells as unchanged fragments and these A9 donors therefore contained stable subchromosomal transferable fragments (STFs). These STFs were then transferred to rhabdomyosarcoma cell line RD. The 11p15 STFs suppressed growth of this line. Using RFLP analysis, the critical region was mapped to a 6 Mb region on 11p15.5. It is intriguing to note that the 500 Kb region encompassing the centromeric end of this region is the same region defined by Weissman's group as containing the WT2

locus and p57 (Reid et al, 1996). Interestingly, in a series of 75 soft tissue sarcomas and 51 Wilms' tumours, analysis of the $p57^{Kip2}$ gene by Southern blot, comparative multiplex PCR, PCR-SSCP, and DNA sequencing failed to reveal any deletions or point mutations (Orlow et al, 1996), further suggestive evidence that the WT2 locus may be the same for Wilms' tumour and rhabdomyosarcoma and is not p57.

Negrini demonstrated that chromosome 11 suppressed tumorigenicity of MCF-7 breast cancer cell line but had no effect on the tumorigenicity of the MDA-MB-231 breast cancer line. Additionally, MCF-7 microcell hybrids that lost the p-arm regained tumorigenicity but remained significantly growth suppressed, this being the third piece of functional evidence for an 11q suppressor gene (Negrini et al, 1994) after the observations in HeLa (Misra et al, 1989) and rhabdomyosarcoma (Loh et al, 1992). Recently, it has been shown that although chromosome 11 does not suppress the tumorigenicity of the MDA-MB-435 cell line, it does abolish the in-vivo formation of metastases (Phillips et al, 1996). Chromosome 11 has also been shown to suppress tumorigenicity of human cell lines derived from cutaneous carcinoma (Conway et al, 1992), lung adenocarcinoma (Satoh et al, 1993), thyroid carcinoma (Yoshida et al, 1994), neuroepithelioma (Chen et al, 1995). Chromosome 11 has also been transferred to ovarian cancer cell lines (see below).

Additionally, chromosome 11 transfer showed no suppression of tumorigenicity of human cell lines derived from colorectal carcinoma (Tanaka et al, 1991) and renal carcinoma (Yoshida et al, 1994). However, inhibition of *in-vitro* growth rate was observed for the colorectal carcinoma cell line (Tanaka et al, 1991).

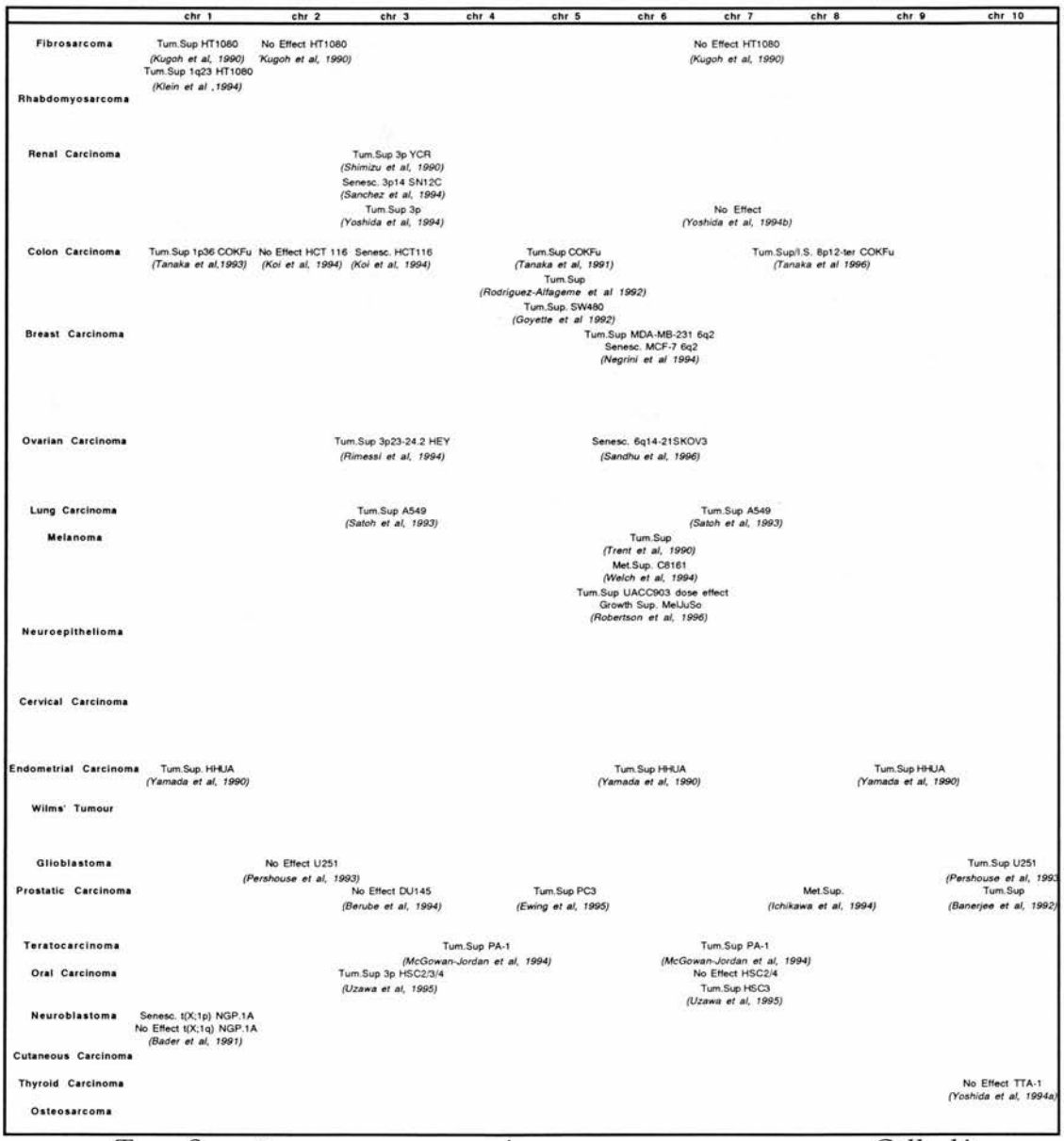
### 1.6.10 Suppression of the cancer phenotype by transfer of other chromosomes

There have been over 130 publications utilising microcell transfer since Saxon's first experiments with MMCT into HeLa. Many of these

have been performed using normal human chromosomes transferred into human cancer cell lines. This information is summarised in table 1.7.

In only one case has the functional approach resulted in cloning of a novel tumour suppressor gene (Ichikawa et al 1992, Dong et al, 1995, see below).

## Table 1.7a Functional analysis by MMCT in different cancer cell lines




*Tum.Sup=tumour suppression. senesc=senescence. Cell line used is given, as is the chromosome fragment when determined.*

## Table 1.7b Functional analysis by MMCT in different cancer cell lines (continued)

| | chr 11 | chr 12 | chr 13 | chr 15 | chr 17 | chr 18 | chr 19 | X-chromosome |
|---|---|---|---|---|---|---|---|---|
| **Fibrosarcoma** | Tum.Sup HT1080 *(Kugoh et al, 1990)* | No Effect HT1080 *(Kugoh et al, 1990)* | No Effect HT1080 *Anderson et al, 1994)* | | | | | |
| **Rhabdomyosarcoma** | Tum.Sup R.D. 11p15 *(Koi et al, 1993)* Growth Sup. A204 11p15, 11q *(Loh et al, 1992)* | | | | | | | |
| **Renal Carcinoma** | No Effect *(Yoshida et al, 1994)* | | | | | | | |
| **Colon Carcinoma** | No Effect COKFu *(Tanaka et al, 1991)* | | | No Effect SW480 Senesc. SW480 *(Goyette et al 199,oyette et al 1992)* | | Tum.Sup. COKFu *(Tanaka et al, 1991)* Growth Sup. SW480 *(Goyette et al 1992)* | | |
| **Breast Carcinoma** | um.Sup.11p/G.S. 11q MCF7 No Effect MDA *(Negrini et al 1994)* | | No Effect MCF-7 *(Casey et al, 1993)* | | Senesc. MCF-7 non p53 *(Casey et al, 1993)* Senesc. MCF-7/MDA = p53 *(Negrini et al 1994)* Tum.Sup.not p53/NM23 *(Rinker-Schaeffer et al, 1994)* Tum.Sup. CAL51 not p53/BRCA1 *(Theile et al,1995)* | | | |
| **Ovarian Carcinoma** | Growth Sup. HOC8 *(Cao et al,1993)* Growth Sup. HEY *(Rimessi et al, 1994)* | | | | | | | Senesc. *(Horikawa et al, 19* |
| **Lung Carcinoma** | Tum.Sup. A549 *(Satoh et al, 1993)* | | | | | | | |
| **Melanoma** | | | | | | | | |
| **Neuroepithelioma** | Tum.Sup. A673 Tum.Sup. TC32 Tum.Sup. SK-N-MC *(Chen et al, 1995)* | | Tum.Sup. A673 Tum.Sup. TC32 Tum.Sup. SK-N-MC *(Chen et al, 1995)* | | Tum.Sup. A673 Tum.Sup. TC32 Tum.Sup. SK-N-MC *(Chen et al, 1995)* | | | |
| **Cervical Carcinoma** | Tum.Sup. t(11;X) HeLa *(Saxon et al, 1986)* Tum.Sup. SiHa *(Koi et al, 1989)* | | | | | | | |
| **Endometrial Carcinoma** | Tum.Sup. HHUA *(Yamada et al, 1990)* | | | | | | Tum.Sup.. HHUA *(Yamada et al, 1990)* | |
| **Wilms' Tumour** | Tum.Sup. *(Weissman et al, 1987)* Tum.Sup. 11p15.5-14 G401 *(Dowdy et al, 1991)* | | No Effect *(Weissman et al, 1987)* | | | | | No Effect *(Weissman et al, 19* |
| **Glioblastoma** | | | | | | | | |
| **Prostatic Carcinoma** | Met.Sup. *(Ichikawa et al, 1992)* Met.Sup. *(Rinker-Schaeffer et al, 1994)* | Tum.Sup. 12q13 DU145 *(Berube et al, 1994)* | | | Met.Sup. *(Rinker-Schaeffer et al, 1994)* | | | |
| **Teratocarcinoma** | | Tum.Sup. PA-1 *(McGowan-Jordan et al, 1994)* | | | | | | |
| **Oral Carcinoma** | | | | | | | | |
| **Neuroblastoma** | Differentiate NGP.1A *(Bader et al, 1991)* | | | | Tum.Sup. NGP.1A *(Bader et al, 1991)* | | | |
| **Cutaneous Carcinoma** | Tum.Sup. A3886TGc2 *(Conway et al, 1992)* | | | | | | | |
| **Thyroid Carcinoma** | Tum.Sup. TTA-1 *(Yoshida et al, 1994)* | | | | | | | |
| **Osteosarcoma** | | | Senesc. *(Banerjee et al, 1992)* | | | | | |

*Tum.Sup=tumour suppression. senesc=senescence. Cell line used is given, as is the chromosome fragment when determined.*

### 1.6.9 Functional cloning of a chromosome 11 metastasis suppressor gene

The only example to date of functional cloning of any tumour suppressor gene was the cloning of KAI1 (kang ai, Chinese for anticancer) by Dong and colleagues (1995). They initially showed that metastatic ability of rat AT6.1 prostate cancer cells was suppressed by fusion with non-metastatic cancer cells (Ichikawa et al, 1991). MMCT of human chromosome 11 suppressed metastatic ability, and the gene was mapped from these hybrids to 11p11.2-13 (Ichikawa et al, 1992). They isolated human genomic DNA fragments from metastasis suppressed rat prostate cancer cells bearing only the 11cen-p13 chromosomal region using human-specific Alu-element mediated PCR (Alu-PCR). The Inter-Alu fragments of unique DNA were then used to screen a cDNA library derived from the same cell line. Five cDNAs were obtained which were expressed in suppressed but not in metastatic hybrids by RT-PCR. One of the sequences designated KAI1 was localised to 11p11.2 by FISH. Northern analysis revealed that KAI1 encoded a 2.4 Kb transcript, and was expressed in non-metastatic rat prostate cancer cells and normal human tissue, but not in the metastatic line and hybrids. KAI1 encodes a 267 amino acid protein with transmembrane domains. KAI1 was cloned into an expression vector and transfected into AT6.1 rat prostate cancer cells, which resulted in suppression of lung metastases in-vivo, inhibition of invasive ability but no alteration of growth rate in-vitro.

KAI1 belongs to a family of membrane glycoproteins called the Leucocyte surface proteins which are thought to function in cell-cell and cell-ECM interactions. Clearly, a functional assay using rodent cells greatly facilitated the cloning of this gene, and represents a paradigm in functional cloning of tumour suppressor genes.

### 1.6.11 Ovarian carcinoma tumour suppression by monochromosome transfer

Ovarian cancer has been the subject of functional analysis using several monochromosomes. In 1993 Cao and co-workers presented a poster showing that they had introduced normal chromosomes 11 and 19 into HOC8, a human ovarian cancer cell line. Whilst chromosome 19 had no effect on HOC8, chromosome 11 prolonged the doubling time of HOC8 and reduced the soft agar colony forming efficiency of these cells (Cao et al, 1993). In-vivo tumorigenicity was not assessed. However, this work remains unpublished after 4 years.

In 1995 another poster was presented by the same group (Cao et al, 1995) this time presenting results of transferring chromosomes 11 and 17 to the human ovarian cancer cell line SKOV-3, and demonstrated that in-vitro doubling time was prolonged for both chromosome 11 and 17 introduction. In-vivo suppression of tumorigenicity in nude mice was observed in 2 of 4 chromosome 17/SKOV-3 microcell hybrids and in 3 of 5 chromosome 11/SKOV-3 microcell hybrids.

Rimessi and colleagues transferred chromosomes 3 and 11 into an ovarian carcinoma cell line HEY (Rimessi et al, 1995).

Chromosome 3 powerfully suppressed tumorigenicity and altered the phenotype of HEY cells. By deletion mapping of hybrids that were not suppressed for tumorigenicity, three regions were found to be involved, two regions in the interval 3p23-24.2 and one at 3p21.1-21.2. These regions concur with the LOH analysis in sporadic ovarian cancers which localised the region to 3p21-p25 (Ehlen and Dubeau, 1990) and do not include the recently cloned FHIT gene (encompassing the fragile site at 3p14.2) involved in renal, gastrointestinal and lung cancer (Ohta et al, 1996; Sozzi et al, 1996). A candidate gene involved in mismatch repair has been cloned from the 3p21-p23 interval (Bronner et al, 1994), but its role in ovarian cancer is not yet determined.

The effect of chromosome 11 introduction into HEY cells was markedly different. Cellular morphology and the immortalised phenotype were unaltered. However, the in vitro growth rate and

clonogenicity in soft agar were reduced. Tumorigenicity was not consistently controlled by chromosome 11 transfer. Although Rimessi and colleagues downgrade the role of chromosome 11 since it does not suppress tumorigenicity in the nude mouse assay, the pattern that they and Cao's group observed might, in fact, be quite consistent with the action of a late-acting tumour progression suppressor which although it does not determine fundamental neoplasia, has more subtle effects not easily detected using the crude assays available, perhaps reflected here in altered growth rate and cloning efficiency, but nevertheless is a powerful determinant of tumour progression. The work of Negrini and colleagues and of Phillips and colleagues (see above) clearly demonstrate that although chromosome 11 does not suppress tumorigenicity of the MD-MBA breast carcinoma cell line, it does suppress the formation of metastases produced by the cell line, arguably as important a clinical endpoint as subcutaneous tumorigenicity. This raises interesting questions about the appropriateness of subcutaneous xenograft tumorigenicity in immunodepleted rodents as the only - or even the major - end point of chromosome transfer experiments designed to look for functionally important tumour suppressor genes, given that the clinical course of cancers may be strongly influenced by genetic events occurring later in the evolution of the disease.

Introduction of the X chromosome seems to carry a functional senescence gene (Horikawa et al, 1996)

Transfer of chromosome 6 also causes senescence of ovarian cancer lines SKOV3, OVCAR3,, whereas chromosome 10 and 14 do not (Sandhu et al, 1996). Sandhu and colleagues went on to show that introduction of the chromosomal fragment 6q13-q21 restored senescence to human and rat ovarian cell lines. They designated this locus SEN6A, distinct from the SEN6 locus located in the interval 6q21-qter which restores senescence to SV40 immortalised human fibroblasts but not to ovarian tumour cells(Sandhu et al 1994). LOH

data implicate three distinct regions of chromosome 6q: 6q14-q15, 6q16.3-q21 and 6q21-q23 in the progression of ovarian cancer (Orphanos et al, 1995).

Another group performing the same experiment transferring chromosome 6 into SKOV-3 and HEY cells did not induce senescence but did completely suppress tumorigenicity and anchorage independence of these cell lines, whereas introduction of chromosome 11q produced no measurable phenotypic change (Wan et al, 1996). It is therefore clear that under differing laboratory conditions, a senescence function may be a non-senescent tumour suppressor in the same cell line, raising the possibility that the earlier definitions of workers may be to some extent merging.

# 2. MATERIALS AND METHODS

## 2.1 DNA preparation

### 2.1.1 Clinical Material

Fresh primary ovarian tumour tissue from 60 patients was transferred directly to dry ice or liquid nitrogen and stored at -70$^0$C until processing. Heparinised blood was obtained from 39 of these patients post-operatively. For the remaining 21 patients fragmented constitutive DNA was extracted from regions of normal tissue from formalin fixed blocks. FIGO staging, histopathology and differentiation state were determined and reviewed in a standardised fashion at a multidisciplinary combined gynaecological oncology clinic. Treatment was planned and delivered in accordance with standard protocols implemented by the multidisciplinary clinic at the time, which consisted of the best possible surgical debulking followed by adjuvant/palliative chemotherapy. The principal chemotherapeutic agents used were chlorambucil, cis-platinum and carboplatinum. Follow-up data on this group are therefore complete and comprehensive. Minimum follow-up is 24 months, and all deaths that have occurred have been due to ovarian cancer. Patient characteristics are outlined in table 3.1(LOH results).

### 2.1.2 Extraction of High Molecular Weight DNA

Up to 3 X $10^7$ Cells were harvested by centrifuging at 1200 rpm for 5 minutes. The supernatant was removed without disturbing the cell pellet. 6 ml reagent B was added to the tube and the cells were resuspended by brief vortexing. The solution was transferred to a 15 ml polypropylene tube, and 45 µl of 50 µg/ml RNAase A solution was added and incubated for 30 minutes at 37$^0$C.

Deproteinisation was performed by adding 1.5 ml 5M sodium perchlorate and shaking the solution on a shaker for 15 minutes at room temperature. The tube was then incubated at 65$^0$C for 25 minutes in a shaking water bath.

DNA extraction was then performed by adding 5.5 ml cold chloroform (stored at -20$^0$C) and placing the tubes on a rotary shaker for 10 minutes. The solution was then centrifuged at 1200 rpm for 1 minute to separate the phases and 800 µl of Nucleon silica suspension was added. The solution was then centrifuged at 1800 rpm for 3 minutes.
The DNA in the top phase was transferred to a fresh tube and centrifuged to remove any remaining silica; the solution was transferred to a second fresh tube. Two volumes of cold Ethanol 100% were added to precipitate the DNA. A Pasteur pipette was sealed and bent using heat and the DNA was spooled out and left to dry on the bench overnight. It was resuspended in 2 ml 1 X TE and the concentration was estimated by spectrophotometry. The final concentration was adjusted to 1 mg/ml.

### 2.1.3 Rapid Miniprep Extraction Of DNA For Microsatellite Analysis

This was conveniently performed using the QIAamp DNA extraction kit (Qiagen):

$10^7$ washed cells were suspended in a final volume of 200 µl PBS.

25 µl Proteinase K stock solution and 200 µl buffer AL were added and the solution was mixed by vortexing.

The solution was incubated for 10 min at 70$^0$C.

210 µl of 100% ethanol was added and the solutions were mixed by vortexing.

The lysate was applied to a QIAamp spin column and centrifuged for 1 min at 8000 rpm.

The solution was washed with 500 µl of buffer AW and centrifuged for 1 min at 8000 rpm and repeated.

200 µl 10 mM Tris-HCl pH 9.0 were added ( preheated to 70$^0$C) and centrifuged for 1 min at 8000 rpm to elute the DNA, which was stored at 4$^0$C after the concentration was estimated using spectrophotometry.

### 2.1.4 Extraction Of DNA From Fixed Archival Material

Extraction of DNA from fixed specimens was performed by cutting 3 x10 μm sections from the block, dewaxing in xylene for 30 min. twice, removing the xylene by washing 3 times in ethanol 100%, and desiccating the specimen under heat. Proteinase K (200 μg/ml) digestion was performed overnight at $37^0$C and then the proteinase K was heat inactivated at $95^0$C for 5 min and debris was removed by centrifugation . The resultant preparation provided adequate DNA template for PCR.

### 2.1.5 Quantification of DNA by spectrophotometry

5 μl of the DNA solution were added to 995 μl TE ( 1 in 200 dilution). Spectrophotometric reading of absorbance at 260 nm (O.D. 260) is proportional to the concentration of DNA in the sample. Absorbance at 280 nm divided by absorbance at 260 nm indicates the purity of the sample. A value of 1.8 is optimal and ratios below this indicate contamination of the sample. an O.D. 260 of 1.0 is equivalent to a DNA concentration of 50 μg /ml, allowing simple proportional calculations to determine DNA concentration of any sample.

## Table 2.1 Chromosome 11 polymorphic microsatellites used

| RH MAP | MAP POSITION | GENETIC DISTANCE | LOCUS | ALF? | NAME | GENBANK | STS TYPE | SIZE | T°an | SEQUENCE | No. of ALLELES |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 28 | -823 | | S569 | | cCI11-434 | | MSAT | 139-157 | 54 | TGT GTA TTA GTC AGG GTT CTC<br>CCT CCA TAA TTC TAT AAG CC | 8 |
| 31 | -806 | | S564 | ALF | cCI11-419 | | ANON | 186 | TD55-50 | GAA GGT TAC GTA GCT TCA CTT AG<br>TTT GCT TTT GCA CAG GAT GTG C | |
| 36 | -744 | -0.37 | S902 | ALF | afm072yd3 | Z16521 | MSAT | 145-163 | 55 | CCCGGCTGTGAATATACTTAATGC<br>CCCAACAGCAATGGGAAGTT | 10 |
| 48 | -599 | -0.27 | S929 | | afm234xc3 | Z17054 | MSAT | 218-240 | 55 | AGGCCCTTCCAAGATCAG<br>CCCAGTTGCCGAACTACC | 12 |
| 71 | -272 | -0.16 | S935 | | afm254zb9 | Z17148 | MSAT | 196-208 | 55 | TACTAACCAAAAGAGTTGGGG<br>CTATCATTCAGAAAATGTTGGC | 6 |
| 74 | -198 | -0.06 | S905 | | afm105xb10 | Z16575 | MSAT | 208-228 | 55 | CAGGCATCTGAAACTTCTTG<br>ATACAGGGGCCAAATAGGTT | 8 |
| 125-127 | 530 | 0.18 | S916 | ALF | afm185ya1 | Z16809 | MSAT | 135-153 | TD57-54 | CAGACTATTCTCATTGCTGC<br>GGACTTCTAAGCCTCCATAA | 9 |
| 131 | 581 | | S533 | ALF | | | MSAT | 300-900 | 60 | GCC TAG TCC CTG GGT GTG GTC<br>GGG GGT CTG GGA ACA TGT CCC C | 10 |
| 138 | 654 | 0.29 | S901 | ALF | afm063yg1 | Z16505 | MSAT | 160-176 | 55 | CCCACATAGATTACTGGCCTC<br>TCCTACATTAGCAGTTGGCA | 8 |
| 147 | 774 | 0.35 | S1358 | ALF | afm345zd1 | Z24577 | MSAT | 138-146 | 56 | ACAACCTGGATGAACCC<br>ACTTTCTGTCTTTATGATTTTGATT | 5 |
| 155 | 847 | 0.39 | S919 | ALF | afm203vg7 | Z16896 | MSAT | 245-261 | 55 | CACCCTAAAACTGTGGATG<br>GGGAAATGTAGCAAGGC | 8 |
| 162 | 909 | 0.41 | S35 | | phi2-22 | | MSAT | 152-162 | TD63-58 | ACA ATT GGA TTA CTA CTA GCA CC<br>AAC TAT GTA TTT GTA TCG ATT AAC C | 6 |
| 161-162 | 909 | 0.41 | S900 | ALF | afm059yc5 | Z16499 | MSAT | 91-109 | 55 | CTATGCCCTCATTAAAACCC<br>GTCGCATCTGTACAATCTGG | 8 |
| 164 | 918 | 0.43 | S940 | ALF | afm268vd5 | Z17194 | MSAT | 163-185 | 55 | TCATCCCCAAATGCTCAG<br>GGAATCAAACTTCACATAGGAGG | 6 |
| 175 | 1041 | 0.47 | S927 | ALF | afm225yb4 | Z17031 | MSAT | 129-149 | TD57-53 | AGTGAGCCGAGTTCGC<br>ACCAAAAGCCTGGAATG | 9 |
| 192 | 1170.5 | 0.58 | S939 | ALF | afm267yh5 | Z17190 | MSAT | 240-248 | 55 | TCAGTTTCCTCATCCACCAA<br>AAAAGCACATATTTTGCTCAGAGTC | 4 |
| 205 | 1282 | 0.63 | S925 | | afm220yb6 | Z17002 | MSAT | 173-199 | 55 | AGAACCAAGGTCGTAAGTCCTG<br>TTAGACCATTATGGGGGCAA | 11 |
| 208 | 1316 | 0.65 | S1336 | | afm286xf5 | Z24048 | MSAT | 232-252 | 58 | TGCAGCAAGCCGAGATT<br>CTGGATGGCATGAGGTTTC | 9 |
| 209 | 1328 | 0.67 | S936 | ALF | afm256za5 | Z17157 | MSAT | 250-256 | 56 | GCAGATATAGGCAGAACTCC<br>CTGTGAAAAGTTACTTGTGGTTG | 4 |
| 213-215 | 1359 | 0.69 | S933 | | afm240ye1 | Z17090 | MSAT | 247-263 | 57 | GGACTCCAGCCTCAGTG<br>GGTGCCTGGTTCACAGTAAA | 9 |
| 221 | 1406 | 0.72 | S934 | | afm248wf5 | Z17119 | MSAT | 180-206 | 58 | GCTGTCCCTGACAACTACATGC<br>TTCCATCAGAACTGGGAATGAG | 11 |
| 223 | 1435 | | S707 | | cCI11-525 | | MSAT | 208 | 55 | CTT AAT GCC ACT GAA CTC TAC C<br>GAA GGA GCC TAA ACT CTT CCA G | TODO |
| 225 | 1452 | 0.77 | S1351 | | afm324zh9 | Z24403 | MSAT | 252-270 | 55 | CAAACCCCAGAAAGATAGA<br>GCAAGTGGAAATACAAAATG | 8 |
| 227 | 1479.5 | 0.78 | S912 | | afm157xh6 | Z16703 | MSAT | 101-123 | TD57-53 | TCGTGAGANTACTGCTTTGG<br>TTTTGTCTAGCCATGATTGC | 12 |
| 233 | 1568 | 0.85 | S910 | ALF | n154yh2(k) | Z16688 | MSAT | 249-261 | 55 | AGCTTTGCAGACAAGGCAAG<br>TCCCTGCTCATAAGTCAGCC | 6 |
| 234 | 1575 | 0.87 | S1320 | | afm234yf10 | Z23726 | MSAT | 225-233 | 56 | AACATTACTAAAAGGTTAAATGAGC<br>ATTAAGGCACCAAATGGG | 5 |
| 235 | 1579 | 0.92 | S969 | ALF | afm205vf10 | Z17333 | MSAT | 141-149 | TD55-50 | TTGATTTGGAAGATTTTCAC<br>GGGGCAGAATGGGTAT | 5 |
| 236 | 1583 | 0.94 | S968 | ALF | afm109xc3 | Z17248 | MSAT | 137-151 | 55 | GGCTCTTGTAGTTTCTTATCTCCT<br>GGCTCTTGTAGTTTCTTATCTCCT | 8 |

Location, primer sequences, PCR conditions, product size, number of alleles and heterozygosity rate of polymorphic microsatellites used. (adapted from James et al, 1994)

## 2.2 Polymerase chain reaction
## Analysis Of Polymorphic Microsatellites By PCR

Oligonucleotides were obtained from the MRC Human Genetics Unit nucleotide synthesis service and from the ICRF oligonucleotide laboratory. The primers were selected on the basis of recently generated microsatellite index maps (Gyapay et al, 1994; Litt et al, 1993; Weissenbach et al, 1992; Couillin et al, 1994) for locus, informativeness and spacing. A recently generated high resolution radiation hybrid map allowed reasonable estimates of physical distances separating these markers (James et al, 1994). Table 2.1 shows all the primers used for polymorphic microsatellite mapping in this project, their locus symbols, their locations, and associated references. For microsatellite analysis of chromosome fragments transferred by microcell mediated chromosome transfer, fluoresceinated primers were used and analysed using the ALF system (see below).

### *2.2.1* Polymerase chain reaction and polymorphic microsatellite detection

Essentially, two standard PCR programmes were used for the microsatellite analysis of patient material:

PCR was performed in a reaction volume of 50 μl under the following reaction conditions: The first programme was ($94^0$C for 3 min) x 1, ($94^0$C for 1 min/$55^0$C for 1 min/$72^0$C for 1 min) x 35, ($72^0$C for 5 min) x 1 in a Hybaid thermocycler. For other primers, a touchdown PCR programme was used:

Reaction mix contained 50 mM K Cl, 10 mM Tris-HCl pH 9, 1% Triton X-100,1.5 mM Mg $Cl_2$, 200 uM deoxyribonucleotide triphosphates, 1.25u Taq polymerase, 10 pmol of each primer, and 100 ng DNA.

### 2.2.2 Checking Of PCR Products By Agarose Gel Electrophoresis

Reaction product was checked on standard 2% agarose run at 100-150 volts in 1 X TAE, stained with ethidium bromide and visualised under UV light.

### 2.2.3 Separation Of Alleles By Denaturing Polyacrylamide Gel Electrophoresis

10 μl of the PCR reaction product was loaded onto 9% or 6% (sequencing) denaturing polyacrylamide gel run in 1 X TBE, and separated by electrophoresis(55 watts for 6 hours for sequencing gels; 300 volts for 5-6 hours for 9% Biorad gels)

Multiple loadings can be performed per track,

### 2.2.4 Passive Transfer Of DNA To Nylon Filter

Separated DNA was passively transferred to Hybond nylon by laying a distilled-water soaked hybond sheet on the polyacrylamide, covering with four sheets of whatman filter paper, and placing a glass plate and weight on top. Passive transfer was allowed to occur overnight. The filter was peeled off the polyacrylamide, washed in 1 X TBE to remove residual polyacrylamide and urea, dried between blotting paper sheets and the DNA was then covalently linked by exposure of the DNA side of the filter to UV light for 4 minutes.

### 2.2.5 Radiolabelling Of DNA For Detection Of Alleles

Detection of the alleles was performed by hybridisation of the filter with $^{32}P$ end-labelled poly(CA) probe. The single stranded $(CA)_{35}$ oligonucleotide was synthesised an 10 pg of this probe was mixed with 50 uCi of gamma $^{32}P$ dATP and 0.3u polynucleotide kinase (Boehringer) and 1 X end labelling reaction buffer (Boehringer) in 20 μl final volume, and incubated for 30 min at $37^0C$.

### 2.2.6 Hybridisation Of Membranes

Pre-hybridisation and hybridisation were performed using the same mix. The hybridisation mix was made up as follows:

| | |
|---|---|
| BSA | 0.5g |
| PVP | 0.5 |
| Ficoll | 0.5g |
| SDS | 1g |
| NaPPi | 1g |
| 20 X SSC | 250 ml |

made up to 1 litre with distilled water.

Pre-hybridisation of the filter was for 10 minutes at $48^0$C The fluid was then replaced with fresh hybridisation mix containing the end-labelled (CA) repeat olignucleotide and the filter was hybridised at $48^0$C for 4 hours. The filter was then washed in 4 X SSC at $48^0$C for 15 minutes four times, and sealed in a plastic bag.

### 2.2.7 Detection Of Hybridisation

The filter was placed in a light-tight cassette with X-ray film and exposed for varying amounts of time at room temperature, and then developed. Two observers visually analysed the autoradiographs and recorded allele imbalance when there was clear reduction in intensity of one allele in tumour DNA. Multiple autoradiographs with different exposure times are usually required since the sensitivity of detecting allele imbalance varies with the intensity of signal. PCR's were repeated at least twice until unequivocal results were obtained. For detailed analysis of the 11q22-q24 region, computerised densitometric analysis was then performed on high-resolution flat-bed scanned autoradiographs using Bio-image whole band analyser software system on a SUN SPARC UNIX platform. The relative ratio of alleles was determined, normalised and compared. Where the tumour allele ratio

differed from the normal allele ratio by 30% or more ($r \leq 0.7$), LOH was assigned.

### 2.3 Statistical Methods

The two-tailed Fisher's exact test was used to determine if there were significant associations between LOH at different loci on chromosome 11 and 17. The same univariate analysis was applied to examine relationships between LOH at any locus and FIGO stage, differentiation grade, histological subtype or survival. Since numerous Fisher's analyses were carried out, the cut-off for significance was set at $p=0.01$, but we have included trends towards significance in the region of $0.07>p>0.01$ where they have supported or suggested biological hypotheses. Kaplan-Meier/ log-rank analysis was performed to determine any relationship between LOH and survival. Multivariate analysis was not attempted since the small initial population precluded further reduction in number by sub-grouping for this type of analysis.

Mann-Whitney U-test was used to compare the means and medians of tumour PR content in populations with and without LOH for the three microsatellite markers. Two-tailed p values were calculated.

Spearman rank correlation was used to examine correlations between tumour ER and PR content in those with and without LOH.

Kaplan-Meier/ log-rank analysis was performed to determine any relationship between allele loss and survival in D11S35 informative subgroups.

Students' t-test and ANOVA were used to compare microcell hybrid and parental control cell lines for functional assays.

### 2.4 Mouse Interspersed Repetitive Sequence PCR (IRS-PCR)

Microcell mediated chromosome transfer is thought to transfer single chromosomes only. This conclusion is derived from karyotypic analysis of microcell hybrids. The possibility was considered that small fragments of mouse chromatin might be co-transferred, since

microcells containing only one chromosome are statistically common only as a result of size fractionation. Two methods were tested to look for murine DNA of reduced genomic complexity. One method, chromosome painting of the microcell hybrid with mouse cot-1 fluorescently labelled DNA is considered later. The other method, outlined here, utilises PCR primers recognising different murine repeat elements which orientate the direction of DNA synthesis in the polymerase chain reaction out of the repeat element and into unique DNA. Using such primer combinations that recognise different repeat elements, a distinct PCR fingerprint is generated from any microcell hybrid containing even small, sub-karyotypic, co-transferred fragments of mouse DNA.

For this method, four such primers were utilised: L1, B1, B2A and B2B. L1 is derived from the murine L1 repeat is the most abundant member of the long interspersed nuclear element (LINE) family, of which there are 70-100,000 copies in the mouse genome. The most frequently found members of the short interspersed nuclear repeat element (SINE) family in the mouse are the B1 (130-180,000 copies) and B2 (80-120,00 copies) repeats. Oligonucleotide primers derived from these sequences have been designed with their 3′ ends directed out of the element and into unique DNA (Cox et al 1991). These sequences are outlined in Table 2.2. Empirically, the best fingerprint patterns were produced using a combination of primers L1 and B1.

**Table 2.2 Sequence of murine repeat PCR primers**

| Locus | Primer sequence | Refs. |
|---|---|---|
| B1 | 5′GTCCGGCCGCCTGGAACTCACTCTGAAG AC 3′ | Cox et al 1991 |
| B2A | 5′TAGACGCGGCCGCTCTTCTGGAGTGTCTG AAGA3′ | Cox et al 1991 |
| B2B | 5′TAGACGCGGCCGCGACTGCTCTTCCGAA GGTCC3′ | Cox et al 1991 |
| L1 | 5′GGACGCAGATGTAGCTGTCTCTTGTGAG AC3′ | Simmler et al 1991 |

The optimal PCR reaction conditions for these primers were:

($94^0$C for 3 min.) X 1;

($92^0$C for 30 sec., $60^0$C for 60 sec., $72^0$C for 3 min) X 35.

## 2.5 Automated Laser Fluorescence (ALF) analysis of microsatellites

### 2.5.1 Method

This method conveniently allows three polymorphic microsatellites to be analysed simultaneously in one loading. One of a primer pair is labelled with fluorescein and a PCR is performed. PCR product is denatured and the sample is loaded onto ALF polyacrylamide gels and electrophoresed. A fixed laser coupled to the gel stimulates the fluorescent PCR products as they reach its fixed position during electrophoresis and the light is detected simultaneously by 40 photodetectors corresponding to the sample lanes. Since two runs of 3 microsatellites can be performed, it is possible to analyse 240 samples in 6 hours. The data is automatically downloaded for analysis by ALF Fragment Manager software.

### 2.5.2 Generating size standards

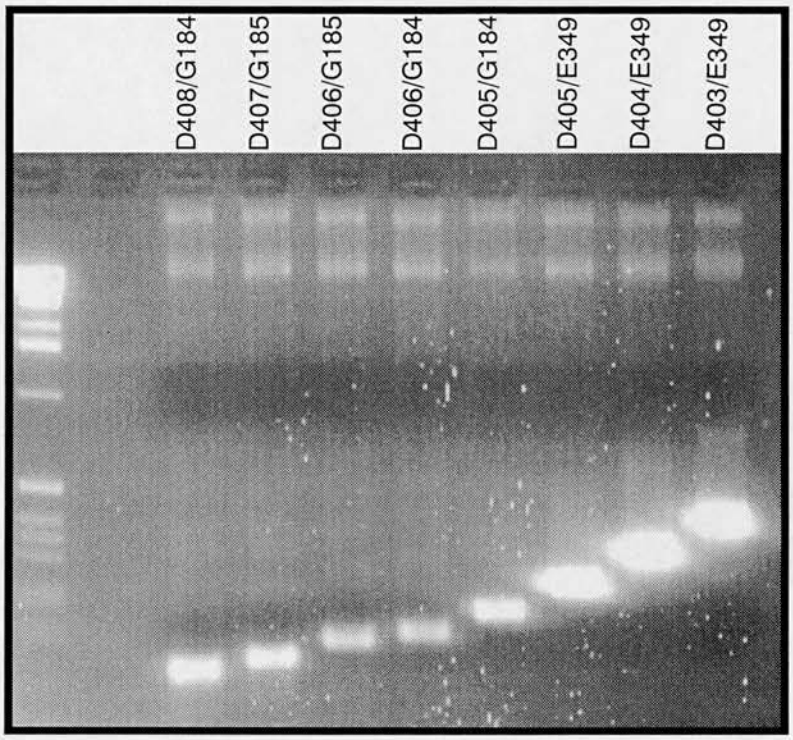
Fluorescent size standards were generated by PCR of M13 DNA template using various M13 PCR oligonucleotide primers (oligonucleotides and M13 DNA were kindly provided by Marian Thompson, MRC HGU) labelled with fluorescent dUTP that define standard sizes of PCR product. The combination of primers used is shown in table 2.3.

**Table 2.3 M13 oligonucleotide primers and expected fluorescent PCR product sizes**

| | G184 | G185 | E349 |
|---|---|---|---|
| D403 | | | 396 bp |
| D404 | | | 311 bp |
| D405 | 175 bp | | 229 bp |
| D406 | 140 bp | 129 bp | |
| D407 | | 104 bp | |
| D408 | 90 bp | | |

The size standards were run on a check agarose gel to confirm the correct size was generated prior to use in ALF polyacrylamide gels (figure 2.1).

**Figure 2.1 Fluorescent size standards for ALF**




### 2.5.3 PCR programmes

A standard PCR programme was used for the fluorescent primers:

($94^0$C for 3 min.) X 1;

($94^0$C for 30 sec., $55^0$C for 30 sec., $72^0$C for 1 min) X 35.

### 2.5.4 Gel casting

The gel cassette was thoroughly cleaned using a nylon brush and non-fluorescent detergent. The plates were rinsed with distilled water. Isopropanol was applied to both inside surfaces of the plates. The top 5 cm of the plates were treated with Bind-Silane:

| | |
|---|---|
| Absolute alcohol | 2 ml |
| Bind Silane | 7.5 µl |
| 10% Acetic acid | 0.5 ml |

made up and used immediately.

The gel cassette was assembled and cast as a 50 ml 6% acrylamide / 7M urea / 1 X TBE gel polymerised using 54 μl TEMED and 210 μl 10% Ammonium persulphate. The comb was fitted and the gel was left to set for 90 minutes.

### 2.5.5 Gel electrophoresis

The gel was inserted into the electrophoresis unit and was clamped tightly ensuring that the laser beam ran across the detectors. 0.6 X TBE was used as running buffer and the gel was pre-warmed for 15 minutes.

0.5 μl of each sample from three PCRs were loaded together with 4 μl Stop buffer containing three appropriate size standards in addition. Electrophoresis was performed at 50 watts for 180 minutes at $40^0$C. The photodetectors sample light every 2 seconds and build an electronic image of light intensity over time corresponding to fragment sizes.

### 2.5.6 Data analysis

Before printing a hard copy of the data, the size standards were horizontally adjusted to super-impose them and delineate small (1-2 bp) size differences accurately between samples.

## 2.6 Enzyme Immunoassay For Quantitative Detection Of Oestrogen And Progesterone Receptor

This assay was performed by technical staff at R.A. Hawkins' laboratory at the Department of Surgery, Edinburgh Royal Infirmary.

Tumour specimens snap-frozen at operation were homogenised in buffer (10 mM Tris, 0.25M sucrose, 1 mM EDTA, pH 8.0, plus 1% v/v monothiolglycerol and 10% v/v glycerol) as previously described (37). After centrifugation at 105,000*g*, the supernatant cytosol was assayed using enzyme immunoassay kits according to the manufacturer's method (ER-EIA and PR-EIA kits, Abbott Laboratories, Maidenhead, Berkshire, UK)(38). The protein content of the cytosol was determined by the method of Bradford (39) and receptor concentrations were

expressed as fmol/mg protein. The PR antibody used was Kd68, a rat monoclonal antibody which recognises both the A and B subunits of PR ( and therefore recognises sequences downstream of codon 165, the start site for subunit A) and binds both free and complexed PR receptor, presumably avoiding the hormone binding site (40).

Recognising that cutoff values for steroid receptor levels are controversial, ER-poor or PR-poor was defined as less than 30 fmol/mg protein and ER-rich or PR-rich was defined as 30 fmol/mg protein or more. These values derive from reports describing clonogenic assays of human ovarian carcinoma cells from clinical specimens, human ovarian cancer cell lines, and clinical work showing that these approximate cut-off values for ER and PR represent a reasonable index of hormone sensitivity (13, 41, 42).

## 2.7 Bacterial Culture And Plasmid Preparation

### 2.7.1 Media And Additives

All media was sterilised by autoclaving prior to use .

*Terrific broth (TB)*

12g Bacto-tryptone, 24g yeast extract, 4g glycerol per 900 ml distilled water. After autoclaving, 100ml solution containing a final concentration of 0.1M potassium dihydrogen phosphate and 0.72M dipotassium hydrogen phosphate was added.

*L-Broth and agar*

2.46 g magnesium sulphate, 10 g Bacto-tryptone, 5 g yeast extract and 10 g sodium chloride per litre of distilled water. For L-Agar, 15 g agar was added per litre of L-Broth

*Ampicillin*

Ampicillin was added to the above media at a final concentration of 50μg/ml to select for bacteria carrying the *amp* antibiotic resistance marker conferred by the plasmid transformed into the bacteria. Ampicillin (Sigma) stock solution was prepared sterile at a final concentration of 50 mg/ml and stored at -20$^0$C.

### 2.7.2 Bacterial Strains

HB101 is a general purpose strain of E. coli used commonly in large scale preparation of plasmids. It was generated in the late 1960's by hybridisation of E. coli K12 and B strains and is highly transformable (Maniatis et al, 1989).

### 2.7.3 Plasmids

Two plasmids were used for their capacity to confer resistance to hmB and to G418.

tgCMV/HgTK (kindly donated by Dr Paul Dickinson), a 5Kb plasmid, was able to confer resistance to hmB under the control of the CMV promoter (Lupton et al, 1991).

pMC1neoPolyA (kindly donated by Dr Julia Dorin) is a 3.8Kb plasmid which expresses the Tn5 *neo* sequence under control of the TK promoter and provides a polyadenylation signal for this gene (Thomas and Capecchi, 1987).

### 2.7.4 Bacterial Transformation

A sterile loop was used to streak out the bacterial host strain from frozen glycerol stock onto L-Agar plate. The next day a colony was used to inoculate 5ml Terrific broth in a universal tube. This was incubated overnight at 37$^0$C on a shaker. 2 ml of this culture was used to inoculate 100ml pre-warmed Terrific broth and the solution was incubated as above until $OD_{550}$ = 0.6 was achieved (usually 1-2 hours). The culture was transferred to a chilled sterile flask and incubated on ice for 20 minutes with gentle swirling to arrest cell division.The cells were transferred to 50 ml tubes and pelleted by spinning for 5 minutes

at 3000rpm at 4$^0$C. The pellet was resuspended in 20 ml ice cold buffer I (30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 50mM manganese chloride, 15% glycerol (v/v), pH5.8 (using filter sterilised acetic acid), stored at 4$^0$C). The suspension was incubated on ice for 15 minutes and thereafter the competent cells were aliquoted into Eppendorf tubes in a dry ice/ethanol bath so that the cells were snap-frozen, thereafter the cells were stored at -70$^0$C ready for heat-shock transformation.

immediately prior to heat shock transformation, a vial of competent cells was thawed out on ice. 0.5μg of plasmid was added to the cells and mixed gently.. The cells were incubated on ice for a further 20 minutes, and then subjected to heat shock by insertion in a 42$^0$C water bath for 90 seconds. The bacteria were then immediately chilled on ice for a further 2 minutes and L-Broth was added to the Eppendorf to a final volume of 1 ml. The Eppendorf was shaken overnight at 37$^0$C and then 100 μl of the culture was streaked out on L-Agar plates containing ampicillin. The plates were incubated overnight in order to provide *Amp$^r$* colonies for large scale plasmid preparation.

### 2.7.5 Plasmid DNA Preparation

(After Maniatis, 1989). A single colony of the transformed Hb101 bacteria grown on L-Agar containing ampicillin was used to inoculate 500ml of Terrific Broth containing 50μg/ml ampicillin and the culture was shaken overnight at 37$^0$C. Next morning the cells were pelleted by centrifugation (6000 rpm at 4$^0$C for 10 minutes) and resuspended in 20 ml GET (50mM glucose, 10mM EDTA pH8.0, 25mM Tris pH8.0). To this solution, a further 20 ml GET was added containing 40 mg lysozyme. The solution was mixed gently and incubated on ice for 30 minutes to promote cell lysis. 80 ml of Alkaline Lysis buffer (0.2M sodium hydroxide, 1% SDS) at room temperature was then added to complete lysis of the cells. The solution was gently stirred with a bacterial loop until well mixed and then incubated on ice for 10

minutes. 60 ml of high salt solution (5M acetate, 3M potassium) was then added to precipitate the unwanted high molecular weight bacterial DNA. The solution was stirred vigorously for 10 minutes, placed on ice for a further 30 minutes and then centrifuged at 10,000 rpm for 10 minutes at $4^0$C without brake. The supernatant was collected, filtered through muslin and 0.6 volumes of isopropanol were added, mixed, and the solution was incubated for at room temperature for 20 minutes. The solution was then centrifuged at 10,000 rpm for 10 minutes at $4^0$C without brake and the pellet was washed with 70% ethanol and dissolved in 10 ml 7.5 M ammonium acetate and incubated on ice for 20 minutes. The supernatant was then centrifuged at 10,000 rpm for 20 minutes at $4^0$C and the supernatant was collected. 2 volumes of 100% ethanol (-$20^0$C) were added and the solution was incubated for 20 minutes at -$20^0$C then pelleted by centrifugtion at 10,000 rpm for 10 minutes at $4^0$C. The plasmid pellet was dried under vaccuum (5 minutes) and resuspended in 1 X TE (10 mM Tris Hcl pH 7.5, 1 mM EDTA). The solution was extracted with phenol, phenol/chloroform, chloroform and the plasmid was re-precipitated using 2 volumes of ethanol and pelleted as above. The plasmid was resuspended in a final volume of 7 ml 1 X TE. 7g caesium chloride and 100µl ethidium bromide (10 mg/ml) were added to give a concentration of 1.55 g/ml. This solution was divided between two quickseal ultracentrifuge tubes and centrifuged for 18 hours at 100,000 rpm in a fixed-angle rotor ultracentrifuge. The covalently closed circular DNA was seen as a band half way up the centrifuge tubeand extracted using an 18 guage needle and 2 ml syringe and poled in a polypropylene tube. The ethidium bromide was extracted using water saturated butan-1-ol, discarding the organic phase repeatedly until it was clear. The plasmid was precipitated by adding 3 volumes of sterile water and 2 volumes of ethanol chilled to $4^0$C and refrigerated for 15 minutes, thereafter centrifuging at 10,000 rpm for 10 minutes at $4^0$C. The pellet was vaccuum dried and resuspended in a volume of 500 µl 1

X TE. The concentration of the plasmid was determined by spectrophotometry.

### 2.7.6 Linearisation Of Plasmid DNA

The plasmids were linearised using the restriction enzyme Xho I which cuts outwith the resistance marker in each of the plasmids. This was done to improve the transfection efficiency of the plasmids. The DNA was purified using the Sigma plasmid purification kit and used for transfection.

## 2.8 Fluorescence *in-situ* hybridisation

Most of the Primed *in-situ* hybridisation and chromosome paint plates presented in this thesis were performed by Dr Viv Watson, and I gratefully acknowledge her help.

### 2.8.1 Metaphase Spreads From Cell Cultures

A confluent culture of cells in a T75 was used to re-seed a T75 as a 1 in 5 dilution in 12.5 ml. The flask was incubated until 60% confluent. colcemid was added at a concentration of 100 ng/ml final concentration was added and the flask was incubated for 5 hours. The cells were shaken off and washed twice in PBS. The cells were pelleted at 1200 rpm for 5 minutes and resuspended in 10 ml hypotonic solution (60 mM K Cl), with the first 4 drops added slowly to prevent clumping, and were then incubated at $37^0$C for 15 minutes. The cells were pelleted once more and resuspended in freshly made fix solution (3:1 methanol: glacial acetic acid), adding the first ml of solution dropwise while vortexing. The cells were stored for 1-5 days at $4^0$C.

The cells were then washed twice in 5 ml fresh fixative. The cells were then resuspended in a small volume of fixatives such that the solution was just cloudy. A single drop of this suspension was dropped from height onto an acid cleaned glass slide that had been moistened with condensation by breathing on it. The suspension was allowed to air dry and observed by microscopy to confirm metaphase spreads. The slides were stored under vacuum for subsequent *in-situ* hybridisation.

### 2.8.2 Primed *in-situ* hybridisation (PRINS)

The slides were dehydrated by passing the slides through an ethanol series of 70%, 90% and 100% (3 minutes each). The slides were allowed to air dry.

PRINS hybridisation mix was made up consisting of 250 ng primer (in 1 μl); 1 μl (10 mM) of each of dATP, dGTP and dCTP; 1 μl (1 mM) dTTP;

1 μl FITC-dUTP; 5 μl PRINS buffer (0.5M K Cl, 0.1M Tris H Cl pH 8.3, 0.015M Mg $Cl_2$, 0.1% BSA); 0.5 μl Taq polymerase (Cetus); 38.5 μl sterile dd$H_2O$. The PRINS hybridisation mix was aliquoted onto a large coverslip and the slide was inverted and allowed to pick up the coverslip by surface tension. The coverslip was sealed with vulcanised rubber solution and allowed to dry in a closed box.

The slides were placed on the thermal cycler and a single denaturing step at $94^0$C for 3 minutes followed by an annealing step of $60^0$C for 3 minutes and a single extension step of $72^0$C for 15 minutes was sufficient.

The slides were immediately transferred to STOP mix (50 mM EDTA, 500 mM Na Cl) at $65^0$C for 5 minutes and then rinsed in 4 X SSC. The slide was then mounted directly in 40 μl Vectashield with DAPI and PI(3.75 μl 100 μg /ml DAPI + 3.75 μl 20 μg /ml PI in 100 μl Vectashield) to produce an R-(reverse) banding-like staining of the chromosomes. The coverslip was sealed with vulcanised rubber solution.

### 2.8.3 Chromosome Painting

Slides were made as above but were allowed to age for 4-7 days before use. The slides were dehydrated through 70%, 90% and 100% ethanol (3 minutes each) and were allowed to air dry. Chromosome paint (STAR*FISH Cambio) was warmed to $42^0$C and mixed well. 15 μl paint was aliquoted for each slide and was denatured by incubating at $65^0$C for 10 minutes. Pre-annealing was performed by incubating at $37^0$C for 15-60 minutes.

The slides were denatured in 70% formamide/ 2 X SSC at 68-$70^0$C for 2 minutes. The slides were quenched in ice cold 70% ethanol and then dehydrated again through 90% and 100% ethanol at room temperature for 3 minutes each. The slides were allowed to air dry.

15 μl paint was placed on a pre-warmed coverslip, and sealed onto pre-warmed slides. The slides were incubated overnight at $42^0$C.

The slides were then soaked in 2 X SSC at $42^0$C until the coverslip floated off. They were then washed twice in 0.5 X SSC: 50% formamide (1;1 mix of formamide + 1 X SSC) at $42^0$C for 5 minutes. The slides were then washed twice in 2 X SSC at $42^0$C for 5 minutes. Finally the slides were mounted in 40 μl Vectashield with DAPI and PI(3.75 μl 100 μg /ml DAPI + 3.75 μl 20 μg /ml PI in 100 μl Vectashield).

### 2.8.4 Microscopy

Samples were initially viewed under ultraviolet light using a Biorad confocal microscope to identify metaphases which show specific hybridisation signals. These metaphases were then scanned using a laser directed through the confocal microscope. The confocal laser scanning microscope contains two photomultiplier tubes enabling signals of two different wavelengths to be collected simultaneously. The two different wavelengths emitted by the FITC and PI can therefore be collected when simultaneously excited by the laser. The channels can be adjusted independently to alter or enhance the signal. The image is sent to a computer screen using biorad software. The two signals can be merged so that the FITC signal for the PRINS or paint probe is superimposed on the PI signal from the chromosome. This gives a composite image of the chromosome and metaphase spread of interest. The images can be stored on an optical disc for future reference.

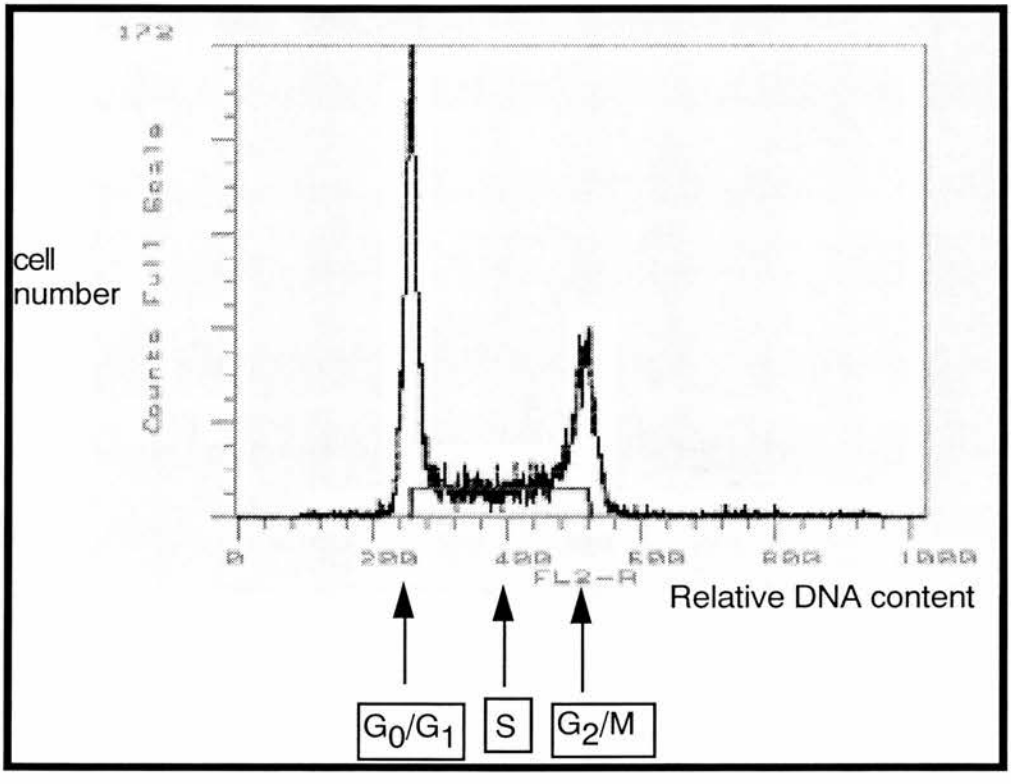
## 2.9 Flow Cytometric Analysis

### 2.9.1 DNA Fluorescence Activated Cell Sorting (DNA-FACS) analysis

A single cell suspension was prepared by trypsinisation. Cells were fixed and stained with propidium iodide to bind DNA, then passed through the flow cytometer. Relative DNA content was assessed and

distribution of the population of the cells with respect to the cell cycle was observed. A typical DNA histogram is shown in Figure 2.2. Cells are predominantly *1n* with respect to DNA content, i.e., most are in $G_o/G_1$. The second peak is for those in $G_2/M$ phase post replication, when cell DNA content is *2n*. relatively few cells exist in S phase.

**Figure 2.2 Typical FACS DNA histogram**




### 2.9.2 FluoroTUNEL technique for Apoptotic Cells

**Principle**

This technique of fluoroTUNEL is an unpublished adaptation of the TUNEL technique developed by Mike Sharrard and set up in our laboratory by Eric Miller.

Programmed cell death (or apoptosis, its morphological equivalent), when established, involves chromatin cleavage as a distinct biochemical feature. Morphological recognition of apoptosis was often difficult and subjective, and for this reason a more objective *in-situ*

method was developed by Gavrieli and colleagues (Gavrieli et al, 1992). This technique became known as TUNEL ( Terminal deoxynucleotide transferase (TdT) mediated deoxy-Uridine triphosphate (dUTP) Nick-End Labelling). This technique measures 3'-hydroxyl ends of DNA fragments generated during programmed cell death. Terminal deoxynucleotide transferase binds to the 3' ends of the fragmented DNA and then generates synthesis of a polynucleotide polymer. Biotinylated or digoxygeninylated deoxyuridine is incorporated at the DNA strand breaks enabling histochemical identification of apoptosis by light microscopy. Second layer FITC labelled anti-digoxygenin antibodies have been used to enable fluorescent detection using FACS(Goryczyca et al, 1994). However, in Dr Sharrard's protocol, there is direct incorporation of FITC labelled dUTP which further simplifies the protocol.

**Method**

$10^6$ single cells were suspended in 1% paraformaldehyde for 10 minutes at room temperature. The cells were pelleted at 500g for 5 minutes and resuspended in 70% ethanol for 10 minutes at room temperature. The cells were pelleted again and washed twice in TBS (.05M Tris buffered Saline pH 7.5). 40 µl of 1 X TdT(terminal deoxynucleotide transferase) reaction buffer added and cells were resuspended for 10 minutes at room temperature. 25 µl TdT reaction mix ( for 100 µl: 20 µl 5 X TdT buffer + 78 µl distilled water + 1 µl TdT + 1 µl Fluorosceinated dUTP) was added, mixed carefully and incubated for 2h at $37^0$C. The cells were then washed twice in TBS and resuspended in TBS. Apoptotic cells are then read on a display of green fluorescence at flow cytometry. Apoptotic cells have high fluorescence and side scatter. Necrotic cells have intermediate fluorescence and low side scatter.

### 2.9.3 Early Apoptosis detection using the FITC-Annexin-V assay

**Principle**

Annexin-V is a calcium and phospholipid binding protein, with a high affinity for phosphlipids. Phospholipids such as phosphotidylserine are normally only found on the inner surface of the cell membrane. One of the earliest features of apoptosis is the loss of cell membrane phospholipid symmetry, resulting in the exposure of phosphotidylserine on to the outer membrane of the cell. Annexin-V binding can therefore be useful in identifying cells undergoing early apoptosis (Vermes et al, 1995). Binding will occur before the cell becomes porous, and therefore if these cells are stained with propidium iodide, they will not take up this dye into their nuclei. Thus, at flow cytometry, three populations of cells can be identified; live, non-apoptotic cells (PI negative, Annexin-V negative); necrotic cells (PI positive, Annexin-V positive); and cells undergoing apoptosis (PI negative, Annexin-V positive). Excitation wavelength was 488 nm. The Annexin-V-FITC signal was detected in channel FL1, and the propidium iodide signal was collected in channel FL2.

**Method**

The Apoptosis Detection Kit (R&D systems) was used for this technique. Cells were washed twice in cold PBS and resuspended in 1 X binding buffer (1 X HEPES / 0.25 mM $Ca\ Cl_2$) at a concentration of $10^6$ cells/ml. 100 µl cells were transferred to a 5 ml tube. 10 µl FITC-Annexin-V and 10 µl propidium iodide were added, gently vortexed and incubated for 15 minutes at room temperature in the dark. 400 µl of 1 X binding buffer was added and the cells were analysed on a flow cytometer immediately.

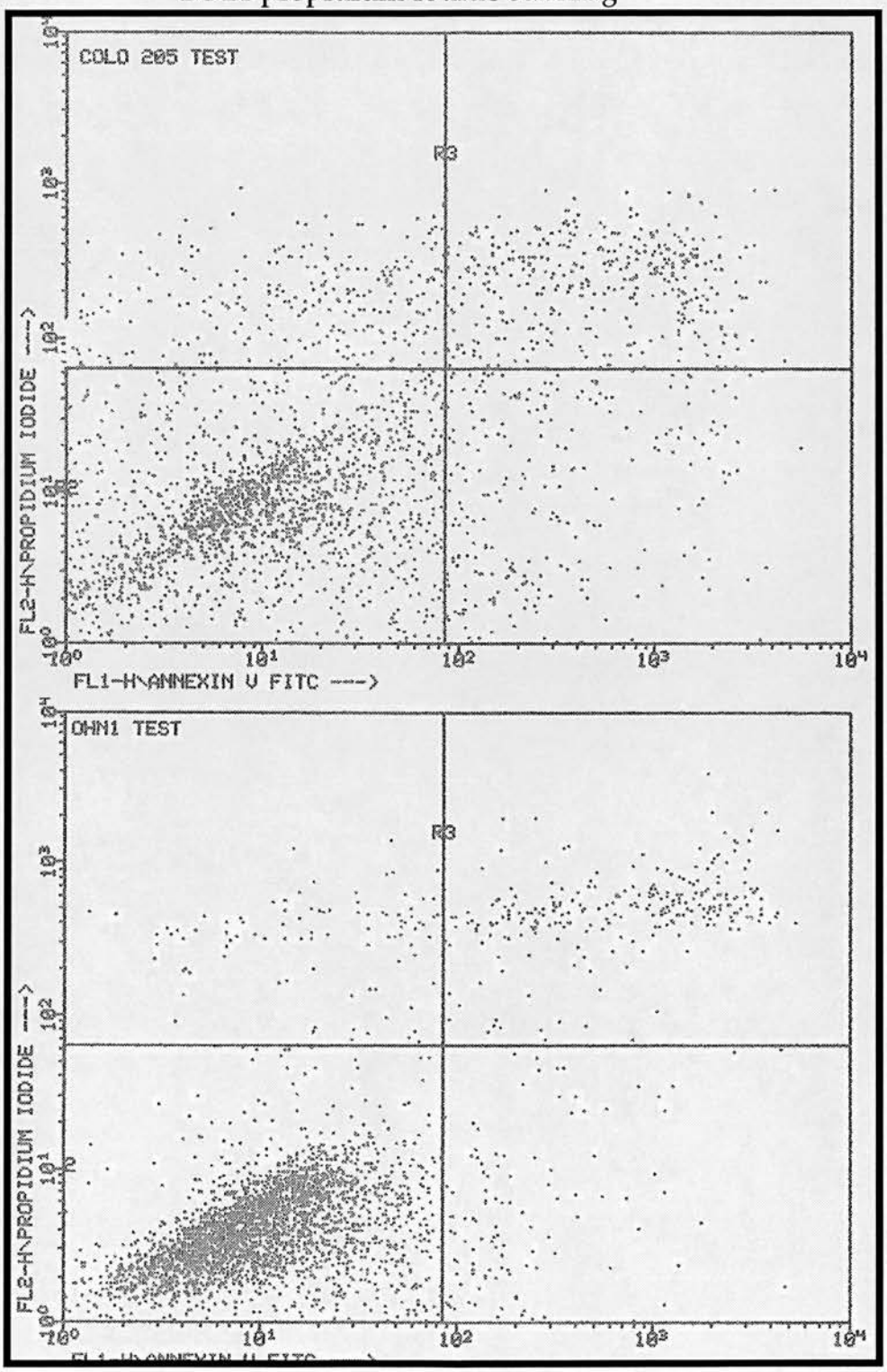
Controls were run of unstained, Annexin-V-FITC only stained, and PI only stained cells.

Annexin-V associated fluorescence registers on the x-axis, propidium iodide fluorescence registers on the y-axis. Thus the bottom left quadrant consists of PI / Annexin V negative cells, i.e. non-apoptotic.

The top right quadrant contains PI / Annexin V positive cells, i.e. necrotic/late apoptotic (one cannot differentiate). The measure of early apoptosis derives from the bottom right quadrant containing Annexin positive PI negative cells.

Figure 2.3 shows typical results obtained with Colo 205 (top), a readily apoptotic colorectal cell line which serves as a positive control. A greater proportion of cells lie in the bottom right quadrant for this cell line than OHN, a *neo*-transfected OH3 parent ovarian cancer cell line (bottom).

**Figure 2.3** **Dual FACS analysis of Annexin-V-FITC and DNA-propidium iodide staining**



## 2.10 Mammalian Cell Culture and Techniques

### 2.10.1 General maintenance

Sterile conditions were maintained routinely. Cell lines were as far as possible maintained on DMEM/10% FCS/P+S as far as possible. Cells were routinely cultured in humidified incubators at $37^0$ C, 5% $CO_2$. Sterile culture flasks, Universal tubes, freezing vials, glassware, and solutions were used throughout. All tissue culture was carried out in a laminar flow hood. Cells were harvested by washing twice in PBS and incubating with a minimal amount of trypsin/versene. Cells were resuspended in 10% FCS media and pelleted at 1200 rpm for 5 minutes for further manipulation.

Cells were frozen in liquid nitrogen, after initial storage for 24 hours at $-70^0$ C, preserved in freezing mix composed of 10% DMSO in FCS. Recovery of the cells was effected by rapid thawing of the vial in a beaker of water at $37^0$ C, followed by two washes in media, and plating in flasks.

### 2.10.2 Chromosome donor cell lines

MCH556.1.5 is a mouse A9-human monochromosome somatic cell hybrid containing a neo-tagged human chromosome 11 as its sole human component (Bader et al 1991, Lugo et al 1987). A9(Neo-1)-4 is a mouse A9-human monochromosome somatic cell hybrid containing human chromosome 1 with integration of pSV2-neo into 1p34-p36 as its sole human component (Koi et al 1989). A9(Neo-12)-4 is a mouse A9-human monochromosome somatic cell hybrid containing human chromosome 12 with integration of pSV2-neo into 12p12 as its sole human component (Koi et al 1989). These three cell lines were maintained in DMEM/10%FCS/penicillin/streptomycin/G418 600 μg /ml.

J1 Cl4 B/B1.22 is a Chinese hamster ovary- human monochromosome somatic cell hybrid containing a single human chromosome 11 as the

sole component which was precisely targeted using homologous recombination into 11q13 (Watson et al 1995).

### 2.10.3 Recipient cell lines

HeLa Ohio TK- was kindly provided by Aaron Cranston and Ivor Hickey, maintained in RPMI/10%FCS/P+S. 278PH, 278HC1, 278HC2 and 278HC3 were derived by lipofection of pTKHyg (conferring hygromycin-B, hmB resistance) into A2780, an ovarian cancer cell line (Eva et al), and were maintained on DMEM/10%FCS/P+S/200 μg /ml hmB.

PEO1 and PEO4 are poorly differentiated ovarian cancer cell lines (Langdon et al 1988) maintained on DMEM/10%FCS/P+S.

OH3 is a cell line derived by lipofection (performed by JEV Watson and K MacLeod)of pTKHyg into OVCAR3, a poorly differentiated ovarian cancer cell line (Hamilton et al 1983), and maintained on DMEM/10%FCS/P+S/hmB 75 μg /ml. OHX is a cell line derived from a SCID mouse xenograft of OH3 and rescued back into cell culture using hmB 100 μg /ml selection. OVCAR4 and OVCAR5 are ovarian cancer cell lines maintained on DMEM/10%FCS/P+S.

### 2.10.4 Lipofection of plasmid DNA into cell lines

This technique utilises Lipofectin (BRL) which is a 1:1 (w/w) liposome formulation of the cationic lipids DOTMA and DOPE in water. This method was used to confer either neomycin or hygromycin resistance to various cell lines, in order to provide clonal lines for further somatic cell genetic manipulation and analysis.

The lipofectin amount was first optimised for each cell line, keeping the amount of DNA and time for transfection constant. After this, transfection was routinely performed.

Typically, $2 \times 10^5$ cells per 60 mm dish in a six-well plate were seeded and incubated for 24-48 hr until 30-50% confluent. Lipofectin and plasmid were allowed to warm up to room temperature for at least 20

minutes prior to use. A similar length of incubation time for DMEM (serum-free and 10%FCS, both antibiotic free) at $37^0$C was allowed before use.

100 μl serum-free DMEM was aliquoted into sterile eppendorfs, two per lipofection.

2 μg plasmid DNA was added to the first eppendorf. The optimised amount of lipofectin is put into the second (range 3-25 μl ). The solutions were then gently mixed in a 5 ml tube and incubated at room temperature undisturbed for 15 minutes. Immediately prior to lipofection, the recipient cultures were washed once in serum-free DMEM. 1.8ml DMEM/5%FCS(no antibiotic) was added to the plasmid/lipid suspension and the 5 ml tube was inverted several times gently. The 2 ml resultant suspension was added to the recipient petri dish and were incubated at $37^0$c for 6-18 hr (depending on optimisation). The suspension was then removed and 4 ml DMEM/10%FCS(no antibiotic) was added and the cells were incubated without selective pressure for at least 48 hr to expand cell numbers. The cells were sub-cultured 1:5 into G418 selective media at previously optimised concentration. Clones were picked by trypsinised using either fine tipped pastettes or ring cloned at 2-4 weeks.

**2.10.5 Selection conditions**

Two exogenous dominant selectable markers were used for positive selection.

The bacterial gene *neo* confers resistance to the antibiotic G418 (geneticin or neomycin) in mammalian cells. *neo* encodes aminoglycoside phosphotransferase which inactivates this antibiotic.

The bacterial gene *hph*, hygromycin B (hmB) phosphotransferase is a gene isolated from *E. Coli* which inactivates hmB, an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* which inhibits protein synthesis by interfering with ribosomal translocation and with

aminoacyl-tRNA recognition. hmB resistance was mediated by the expressing plasmid pTKHyg.

Kill curves for each of these antibiotic were performed for each of the parent un-transfected cell lines prior to introduction of the selectable markers by either lipofection or microcell mediated chromosome transfer.

### 2.10.6 Picking of resistant clones

After selection of cells using the above antibiotics and under conditions that completely kill parallel controls, resistant clones arising from single cells were picked at between 2 and 6 weeks after imposition of selection. When colonies reached over 1 mm in size, the flask was washed with PBS . The top of the flask was removed with a hot probe apparatus. The tip of a fine tipped pastette was half filled with trypsin/versene and applied to the clone, pipetting gently up and down until the cells were dislodged and then transferred to 24 well plates containing 500 µl selection media. The next morning, the media was replaced by 1-2 ml media with the appropriate selection antibiotic at the appropriate concentration for that cell line.

## 2.11 Microcell Mediated Chromosome Transfer

This technique was developed based on two different methods, one by Stanbridge, the other by Stubblefield. Development of this method was a protracted process outlined in the results section, and the method presented here represent optimal conditions for our laboratory.

### 2.11.1 Preparation Of Donor Cell Lines

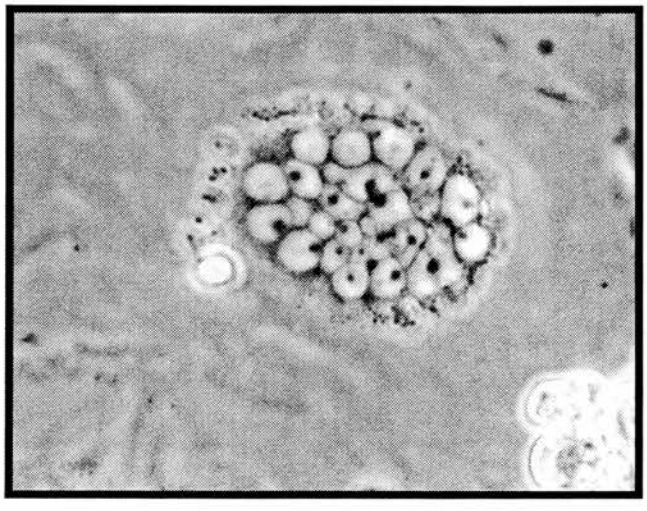
The cell lines used were rodent cell lines with apparently normal human monochromosomes. (see table)

MCH556.1.5 is a mouse A9 cell line containing a human chromosome 11 neo-tagged at 11q14-q22 (Obtained from Aswin Menke, Molecular Carcinogenesis, Silvius Laboratory, Leiden with agreement from Eric Stanbridge, Univ of California at Irvine). 12-neo is a mouse A9 cell line

containing a neo-tagged human chromosome 12 (Kindly provided by Carlo Conti, MD Anderson Cancer Center, Texas with agreement from Carl Barrett, NIEHS, Bethesda ). 1-neo is an A9 mouse cell line containing a neo-tagged human chromosome 1(Kindly provided by Rosemary Bayne, MRC Human Genetics Unit, with agreement from Carl Barrett, NIEHS, Bethesda). J1 Clone4 is a Chinese hamster ovary cell line carrying a human chromosome 11(Fa Ten Kao). The neo-tag in this line was introduced using rodent repetitive sequence mediated targeting to chromosome 11q23(Watson et al) (Kindly provided by Vivienne Watson, ICRF Medical Oncology Unit, Edinburgh). These cell lines were maintained on RPMI or DMEM, 10% FCS, with penicillin and streptomycin and G418 to maintain the neo resistance marker(concentrations of G418 between 400 and 600 μg /ml were used.

The donor cell lines were seeded in 6-12 X 25 $cm^2$ flasks. Colcemid(Demecolcine, Sigma) was added when they reached 70% confluence and micronucleation of the cells was produced by exposure to various concentrations of colcemid for various time periods optimised for each cell line, along the lines of a method developed by Stanbridge and colleagues. Micronucleation of the donor cell line was assessed visually (see Figure 2.4)

**Figure 2.4: Micronucleation of J1 Cl4**
**(75 ng colcemid for 48 hours)**

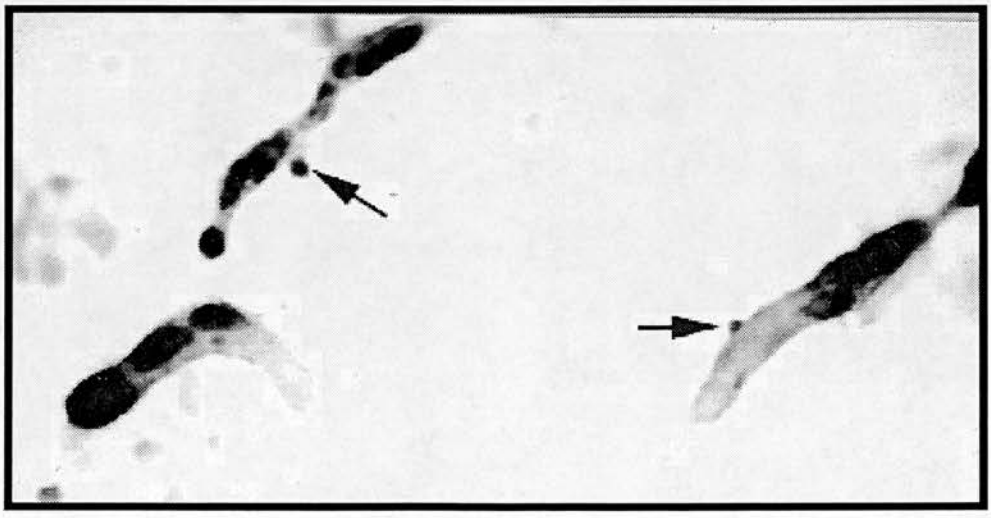


After adequate micronucleation, the donor cells were trypsinised for microcell formation.

### 2.11.2 Formation Of Microcells

The object of the second part of the protocol is to produce microcells, which are plasma membrane coated structures containing a single chromosome, which can then be transferred into the recipient cell line. A technique developed by Stubblefield and colleagues was used for this part of the protocol. This method entails the use of centrifugal force(19,000 rpm for 70 minutes) and the actin filament inhibitor cytochalasin B (Aldrich, 20ug/ml final concentration, prepared as 2 mg/ml stock solution in DMSO)to extrude the microcells out of the micronucleated donor cells on a Percoll(Pharmacia) : 10% FCS/DMEM, 1:1 gradient at $34^0$C. The Percoll solution was adjusted to a hypotonic final concentration (68 mM Na Cl) and was buffered at pH 7.2 (22.5 mM HEPES); cytochalasin mediated extrusion is an active process. This process is graphically demonstrated in figure 2.5 which shows extrusion of microcells by budding from the long actin-inhibited protrusions from the donor cells.

**Figure 2.5 budding formation of microcells (Giemsa stained)**

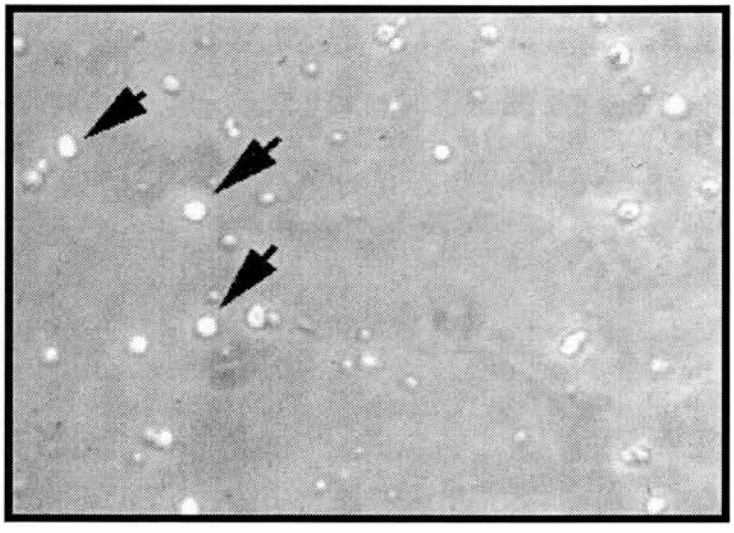


Centrifugation was performed in sterile Oak Ridge tubes using an SS34 rotor in an RC5B centrifuge (Sorvall) as above. The percoll/media

gradient was then transferred out of the tubes and diluted with three volumes of serum free DMEM.

This crude preparation consisting of karyoplasts and microcells was then filtered through a 3 um pore size polycarbonate filter on a dual membrane stirred cell holder (Nuclepore). The filtration step ensured that the maximum size of 3 um limited the size of the microcells and therefore the average number of chromosomes per microcell to one (see Figure 2.6).

**Figure 2.6 Size fractionated Microcells**



### 2.11.3 Microcell Fusion into Recipient Cell Lines

The third part of the protocol consists of the delivery of these single chromosomes to a recipient monolayer cell line. The recipient cell line was seeded 24 hours previously in a 6 cm round dish such that it achieved 50-70% confluence at the time of transfer. The filtered microcell preparation was centrifuged at 1300 rpm for 15 minutes at room temperature to pellet the microcells. The supernatant was carefully removed and the microcells were gently resuspended with a broad tipped pastette in 2.5 ml sterile Hanks balanced salt solution/25 mM HEPES pH 7.2 and 100 μg /ml Phytohaemagglutinin-P (PHAP, sigma). The microcells were layered on the recipient monolayer and

left to attach undisturbed for 20 minutes, the lectin PHAP mediating attachment of the microcells to the recipient. At the end of this incubation, the microcell suspension was aspirated. Pre-warmed ($37^0$C) 2 ml 50% w/v polyethylene glycol in 75 mM HEPES (PEG, sterile, fusion tested, Boehringer) was added for between 60 seconds and 90 seconds depending on the recipient cell line to fuse the lipid membranes of the microcells and recipient cells. The monolayer was then washed 3 times with serum free DMEM and left in non-selective media (10% FCS, pen/strep) for three days to allow the cells to recover and to expand the cell numbers. The cells were then trypsinised and re-seeded into a T175 flask for 24 hours in 50 l non-selective 10% FCS media. Selection was then applied at concentrations of G418 (geneticin) known to completely kill that recipient over a period two to four weeks. The flasks had fresh media replaced twice per week until all the control monolayers had died. At that time, clones present in the microcell flasks were noted, and allowed to expand to a sufficient size to be picked (usually 3-6 weeks). At the appropriate time, the clones were picked according to the picking protocol (see above). DNA was made for microsatellite analysis at the earliest possible time (passage 2-4, from duplicate 24 well confluent plate), and each microcell hybrid clone was bulked and frozen in liquid nitrogen in freezing-mix by passage 4-5.

## 2.12 Histology

### Giemsa Staining

Concentrated Giemsa solution was diluted 1 in 5 with distilled $H_2O$. Cells were dropped onto a clean slide and fixed in 95% ethanol. Cells were Giemsa stained in a slide carrier for 10 minutes. The specimen was dehydrated through an ethanol series of 70%, 90%, and 100% ethanol for 3 minutes each.

## 2.13 Growth Curves

### 2.13.1 Setting up

Log phase cultures were harvested and $10^4$ cells were seeded in 24 well trays. The media was changed 24 hours prior to the first harvest and every 3 days thereafter. Cell counts were harvested every 2 or 3 days depending on the cell line up to 21 days in some cases.

### 2.13.2 Harvesting Cells

The medium from the wells to be counted was removed, and the cells were washed twice with 250 µl PBS. 250 µl trypsin/versene was added and the cells were left for 15 minutes to completely detach. The cells in each well were syringed 3 times and 200 µl of the cell suspension was transferred to one counting pot (see below).

### 2.13.3 Use of the Coulter Counter

The Coulter counter was allowed to warm up for 15 minutes. The orifice tube was rinsed with saline. A background count was taken to ensure that the counter is working properly (background counts >20 suggest caution). The sample was then counted by aliquoting 9.8 ml saline to a counting pot and adding 200 µl trypsinised syringed single cell suspension, mixing and sampling counts from each pot three times. After sampling, the coulter counter was rinsed with saline.

### 2.13.4 Analysis

Cell counts were plotted against time either on logarithmic or linear plots allowing comparisons between parent/control cell lines and microcell hybrids.

## 2.14 Quantitative Adhesion Assay

This assay quantitates the ability of cells to attach to various components of extra-cellular matrix or basement membrane protein, on plastic in a 96 well tray. Non-specific binding was blocked using albumin. The proteins were diluted in PBS to 2-50 µg /ml. 50 µl was

added to quadruplicate wells and the tray was incubated overnight at $4^0$C. The plate was then washed twice with PBS to remove unbound protein. 200 µl of 0.1% w/v BSA in PBS/0.1% azide (WaB) was added to each well immediately, and incubated at $37^0$C for 1-2 hours.

Late log-phase cells were harvested and syringed to form a single cell suspension. The cells were resuspended in standard 10%FCS medium. 110 µl of 10X Hanks buffered salt solution was added to a pot of radioactive chromate (Sodium Chromate[$^{51}$Cr], Amersham). 100 µl of this mix ( 90u Ci) was added to the cells, mixed well and incubated at $37^0$C in a lead pot, resuspended every 10 minutes. After 60 minutes, the cells were washed 3 times in serum free media. A count of known number of cells at the last wash was taken, and 10 µl 0.4% trypan blue due was added to determine cell count and viability. The cells were resuspended at 2 X $10^5$ viable cells per ml in serum free media.

Plates were washed twice in PBS to get rid of traces of azide. After removing PBS, plates were placed on ice. Labelled cells were immediately added (50 µl, $10^4$ cells per well). Cells were incubated for 30 minutes to 6 hours at $37^0$C. After the allotted time the plates were gently washed to eject unbound cells then slowly immersing the whole plate into PBS supplemented with cations (1 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$)and the amount of radioactivity remaining was counted. The percentage adhesion was calculated by knowing how much radioactivity was put into each well initially.

**2.15 MTT Assay**

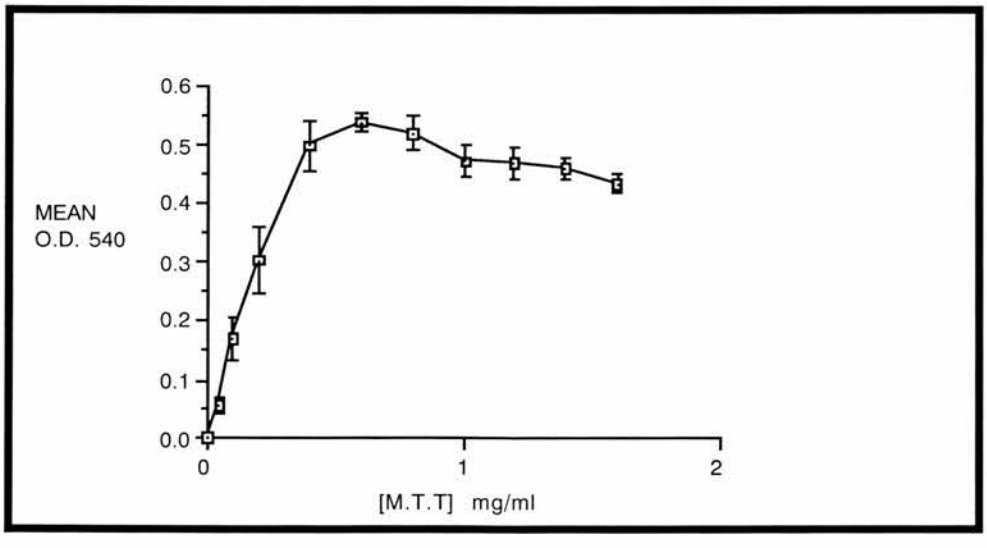
The matrigel invasion and migration assays outlined below both depended on the MTT assay in order to quantitate the differences between microcell hybrid clones. Development of the MTT assay is outlined in this section.

An initial MTT toxicity curve was generated to determine the optimal MTT concentration for the 3 hour incubation. 5 X $10^3$ cells were plated in each well of a 96 well plate. MTT was made up as a filter sterilised solution in PBS (2 mg/ml) and wrapped in foil (MTT is light sensitive). 24 hours later MTT at a range of concentrations between 0.05 and 1.4 mg/ml was added to the 96 wells, and incubated for 3 hours after wrapping the plate in foil.

A computerised plate reader to read absorbance at 540 nm was allowed to warm up for 15 minutes prior to use.

The 96 well plates were unwrapped and MTT was aspirated to a burnable waste container. 200 µl Spectroscopic grade DMSO was added and the content of the well was mixed to solubilise MTT. The OD at 540 nm was then read immediately and the data was output to a simple output data display program. Data was printed out and the peak concentration indicating activity at that cell number was observed from a graph plotting concentration of MTT versus OD at 540 nm (figure 2.7).

**Figure 2.7: Optimisation of MTT concentration for OVCAR3**




The plate was disposed of in a sawdust filled bag to a burnable waste bin.

### 2.15.1 Matrigel Invasion Assays

This assay is based on the principle that basement membrane plays an important part as a barrier against tumour cell invasion.

The invasion assay measures the ability for cells to invade through a matrigel layer in vitro. It is necessary to inhibit protease inhibitors present in serum in order to allow invasion to occur. Therefore FCS was pre-treated by lowering the pH to 3.0 for 2 hours with H Cl and then neutralised to pH 7.3 with Na OH.

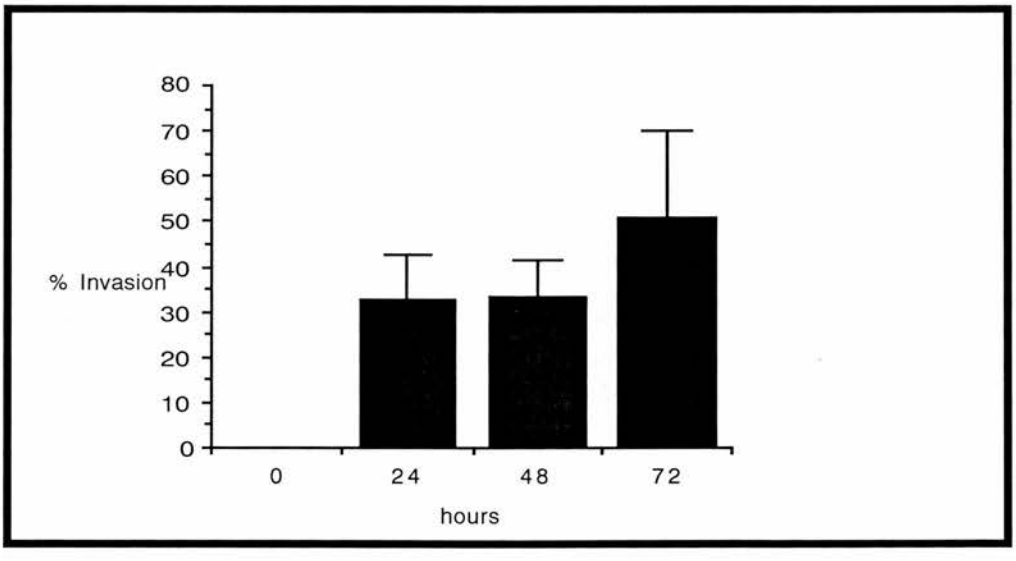
Pre-aliquoted Matrigel (Beckton Dickinson) was thawed on ice and diluted 1:5 in ice cold pre-treated culture medium at a final concentration of 1 mg/ml.

The matrigel layer was formed in Transwell cell culture chambers (Costar) which consist of 12 well plates with inserts containing a porous Nuclepore 12 um polycarbonate membrane. The chamber's margin at the interface with the filter was treated by rubbing a fine layer of paraffin wax to provide a water-repellent treatment and prevent meniscus formation thereby ensuring even matrigel thickness over the surface of the polycarbonate membrane. The membrane was wetted with cold culture medium and re-aspirated with a fine-tipped pastette. 140 µl of cold diluted matrigel was aliquoted into each transwell and even distribution of the extracellular matrix was ensured by tilting. The matrigel was allowed to gel by incubating at $37^0$C for 30 minutes.

The cells to be used were trypsinised and washed 3 times in with cold culture medium and resuspended at a concentration of $10^5$ cells per 0.5 ml. 1 ml culture medium was aliquoted into the lower compartment and 0.5 ml($10^5$) cells were dispensed into the upper compartment. The transwell plate was incubated in a humidified incubator for a varying time course in order to optimise the time for the invasion assay.

The number of cells on the upper and under-surface of the porous membrane were assessed using the methyl toluene test (MTT) assay (see below). 50 and 100 µl of MTT stain were added to the upper and lower compartments of the transwell respectively and incubated for 3 hours. The crystals were removed from the under-surface of the porous membrane using a 1 cm diameter circular whatman filter paper, and the crystals were solubilised by immersing it in 1 ml DMSO. The medium was decanted from the upper chamber and the transwell was placed in a culture well containing 1 ml DMSO, and rotated on a clinical specimen rotator. 200 µl aliquots of solubilised crystals were transferred to an ELISA plate and read at 570 nm on a spectrophotometer. Invasion was determined as the ratio of the under-surface A570 reading relative to the upper surface A570 reading.

**Figure 2.8 Invasion of OVCAR3 in matrigel: time course**




On the basis of the above experiment (figure 2.8) I routinely used 48 hours for OVCAR3 invasion in matrigel.

### 2.15.2 Transwell migration assay

The purpose of this assay was to quantify the haptotactic response of the ovarian cancer cells to ECM components, i.e. observation and

quantification of ECM component directed stimulation of cellular migration. This assay allows assessment of the relative contribution of the components to stimulation of migration and also allows an assessment of the effect of chromosome 11 introduction on this process.

Extracellular matrix component substrates (i.e. fibronectin, laminin, collagen IV) were immobilised on the lower surface of the polycarbonate membrane by incubating the under-surface of the 8.0 μm pore-size transwell cell culture insert (Costar) in a 24 well plate with 250 μl of 10 μg /ml solution of the matrix component at 37$^0$C for 1 hour. The transwells were blocked by transferring the transwell to a 24 well plate with BSA 0.1% for 1 hour at room temperature. The transwell under-surface was washed twice by replacing the BSA with PBS. 400 μl serum-free DMEM were applied to the lower compartment. 5 X 10$^4$ cells in a final volume of 100 μl serum-free DMEM were applied to the top compartment of the transwell. The cells were allowed to migrate across the membrane at 37$^0$ C for 72 hours. Non-adherent cells were removed by rinsing the upper chamber with PBS X 2. 10 and 40 μl of 2 mg/ml MTT were respectively added to the top and bottom compartments of the transwell and incubated for 3 hours at 37$^0$ C. Colorimetric analysis was performed (see above) and the percentage migration was quantified at compared with control plates (transwells coated with 0.1% BSA only on their under-surface).

### 2.16 Soft Agar Clonogenicity Assays

A 5% Seaplaque agar solution was made in distilled water and autoclaved.

Soft agar plates were formed using an underlay consisting of 0.5% Seaplaque agar (at 50C) made up in DMEM/20% FCS (5 ml per dish) and left to set for 20 minutes. The cells were overlaid in a 0.3% agar/media mix.

Log-phase cultures of the cell lines to be tested were trypsinised and single cell suspensions were formed by springing the cells through an 18 gauge needle.

The cells were resuspended at a volume of 5 ml and a concentration of 5x final desired plating density in a 25 ml tube. Final cell densities of $10^4$ cells per plate were used.

23 ml medium was aliquoted into a second tube and warmed to $37^0$c for 20 mins. 2 ml 5% agar was added to the 23 ml medium and 20 ml of this was immediately aliquoted to the tube containing the cells (to give a final concentration of 0.32%). The cells were mixed quickly but gently avoiding bubbles and 2.5 ml of the cell/agar mix into each of 8 petri dishes. The plates were agitated to ensure even spreading without bubbles. The dishes to set before moving (20 min) to avoid satellite colonies. 1 ml media was overlaid on to the plate over the set agar and incubated at $37^0$c. The media was changed weekly. The colonies for 8 plates per cell line were counted at 2 weeks by scoring the colonies (>50 cells) at low power to calculate the colony forming efficiency (CFE) for each cell line.

**2.17 Xenograft assay**

Cells were grown in T175 flasks and were harvested by trypsinisation and pooled. Varying cell numbers generally between $10^6$ and 4 X $10^7$ cells (depending on the minimum cell number required) were aliquoted, pelleted and resuspended in 100-500 µl serum free media, transported on ice and sub-cutaneously injected within 3 hours into either nude or SCID mice depending on the cell line. Tumour volumes were calculated weekly based on bi-dimensional tumour diameter measurements.

### 2.17.1 Matrigel

Matrigel(Beckton Dickinson) is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (which produces copious extracellular matrix protein). It contains laminin, collagen IV, heparan sulphate proteoglycans, entactin and nidogen. Additionally it contains TGF-beta, FGF, tissue plasminogen activator, and other growth factors. Matrigel sets rapidly at physiological temperatures above $22^0$C and must be kept at below $4^0$C until injection. Matrigel promotes cell attachment and invasion and can therefore promote xenograft formation in the mouse xenograft assay.

In those cell lines that did not grow using the standard method, the cells were pelleted and resuspended in 250 µl 10%FCS media stripped of protease inhibitors, mixed with 250 µl Matrigel transported on ice and injected into SCID mice as above. In some experiments where cell lines were known to increase their rate of proliferation in response to oestradiol, pellets were depot injected 48 hours into the mice prior to cell injection.

### 2.17.2 Recovery of Cell Lines from Xenografts

Xenografts were recovered into PBS in a universal under sterile conditions. In a laminar flow hood, the xenograft was finely minced in 5 ml media using two sterile scalpel blades. The fragments were transferred to a universal and roughly pipetted using a broad-tip pastette to further dis-aggregate the xenograft. The cells were returned to a 10 cm round dish with fungizone and, where appropriate, a positive selection agent if a marker had been previously introduced, to facilitate rapid deletion of background stromal mouse cells (see above).

# 3. LOSS OF HETEROZYGOSITY ANALYSIS

The cytogenetic and LOH( or allele imbalance) findings suggesting several disrupted chromosome 11 regions in ovarian cancer (see introduction) prompted the first experimental component, whereby I repeated analysis of the major regions previously shown to be involved and extended the LOH analysis into the sub-telomeric portion of 11q which was not previously examined.

### 3.1 Whole chromosome 11 LOH analysis

The initial study used 10 polymorphic (CA) repeat microsatellites to screen a population of patients with ovarian tumours. These polymorphic markers are outlined in Table 3.1:

**Table 3.1 Polymorphic microsatellites: identity and location**

| Locus | Location | Name | Reference |
|---|---|---|---|
| D11S922 | 11p15.5 | AFM217yb10 | 1,2 |
| D11S569 | 11p15.3 | cCIII-434 | 2,3 |
| D11S935 | 11p13 | AFM254zb9 | 1,2 |
| D11S905 | 11p13-12 | AFM105xb10 | 1,2 |
| D11S873 | 11q14.3 | Mfd127 | GDB ID no. 32638 |
| D11S35 | 11q22 | phage2-22 | 2,4 |
| D11S897 | 11q23.1 | Mfd231 | GDB ID no. 34742 |
| D11S925 | 11q23.3 | AFM220yb6 | 1,2 |
| D11S912 | 11q24.1 | AFM157xh6 | 1,2 |

*(Refs. and abbreviations: *1= Weissenbach et al., 1992; Gyapay et al., 1994; Couillin et al 1994. 2=Litt et al., 1993. 3=Phromchotikul et al 1992. 4=Litt et al 1990. GDB=genome data base)*

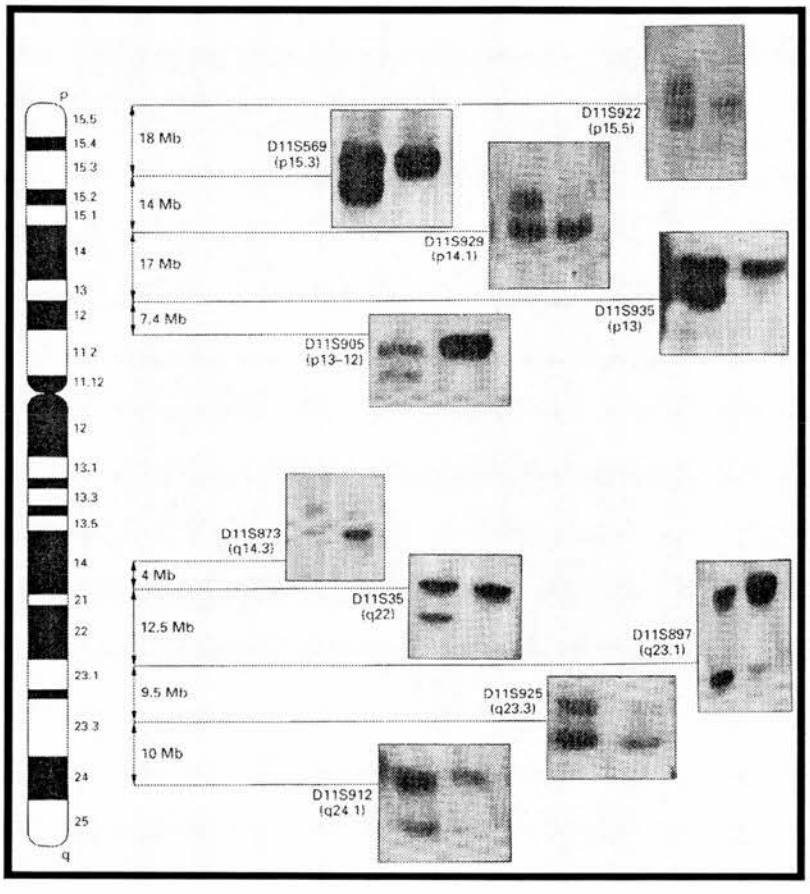
Molecular analysis of blood-tumour pairs from 60 patients with ovarian tumours were used for this part of the analysis. The clinico-pathological characteristics of the patient cohort are outlined in Table 3.2. The findings for stage and histology were validated within the Edinburgh multidisciplinary combined gynaecological-oncology clinic consisting of site-specialised pathologists, gynaecologists and oncologists, ensuring rigorous quality control of the data.

**Table 3.2: Clinicopathological characteristics of 60 patients with ovarian tumours**

| | | |
|---|---|---|
| **Number of patients** | | **60** |
| *Ovarian adenocarcinoma* | | **47** |
| **Histology** | | |
| Serous | 25 | |
| Endometrioid | 14 | |
| Mucinous | 5 | |
| Clear cell | 3 | |
| **Differentiation** | | |
| Well | 3 | |
| Moderate | 14 | |
| Poor | 25 | |
| Not known | 5 | |
| **Stage** | | |
| I/II | 16 | |
| III/IV | 29 | |
| Not known | 2 | |
| **Surgical Treatment** | | |
| Completely Debulked | 32 | |
| Incompletely Debulked | 15 | |
| Not Known | 2 | |
| **Chemotherapy** | | |
| Chlorambucil | | |
| Adjuvant | 3 | |
| Palliative | 6 | |
| Cis-platinum | | |
| Adjuvant | 11 | |
| Palliative | 8 | |
| Carboplatinum | | |
| Palliative | 2 | |
| None | 13 | |
| **Borderline Malignant Potential** | | **5** |
| **Mixed mesodermal tumour** | | **2** |
| **Granulosa tumour** | | **2** |
| **Teratoma** | | **1** |
| **Benign adenofibroma** | | **3** |

Examples of allele loss for each of the markers are shown in Figure 3.1.

**Figure 3.1: Examples of LOH analysis**




*Idiogram of chromosome 11 with examples of LOH at each of the loci. Constitutive DNA(left lane) and tumour DNA(right lane) for each microsatellite locus are shown with approximate distances between loci*

Loss of heterozygosity was observed with variable frequency using different markers and analysing the many heterogeneous subgroups of ovarian cancer. LOH results for all markers and subgroups in this study are summarised in Table 3.3.

**Table 3.3 Loss of heterozygosity rates for all subgroups**

| LOCUS | ALL TUMOURS | ADENOCA. | SEROUS | ENDOMETRIOID | MUCINOUS | EARLY FIGO | ADVANCED FIGO |
|---|---|---|---|---|---|---|---|
| D11S922 | 24/47 (51%) | 16/36 (44%) | 11/20 (55%) | 4/11 (36%) | 0/2 | 3/12 (25%) | 11/22 (50%) |
| D11S569 | 23/43 (54%) | 14/30 (47%) | 9/19 (47%) | 3/5 (60%) | 1/4 (25%) | 4/9 (44%) | 10/20 (50%) |
| D11S929 | 13/47 (28%) | 9/38 (24%) | 3/20 (15%) | 3/11 (27%) | 1/4 (25%) | 3/13 (23%) | 5/23 (21%) |
| D11S935 | 18/50 (36%) | 13/39 (33%) | 4/18 (22%) | 6/13 (46%) | 2/5 (40%) | 4/15 (27%) | 8/23 (34%) |
| D11S905 | 21/45 (47%) | 15/33 (45%) | 7/16 (44%) | 5/10 (50%) | 1/5 (20%) | 6/14 (43%) | 9/18 (50%) |
| D11S873 | 11/25 (44%) | 9/22 (41%) | 5/12 (42%) | 0/5 | 2/3 (66%) | 4/7 (57%) | 5/14 (36%) |
| D11S35 | 16/50 (32%) | 14/39 (36%) | 9/23 (39%) | 0/9 | 2/4 (50%) | 4/11 (36%) | 9/26 (35%) |
| D11S897 | 13/40 (32%) | 9/32 (28%) | 6/18 (33%) | 1/6 (17%) | 1/4 (25%) | 3/12 (25%) | 6/19 (32%) |
| D11S925 | 24/45 (53%) | 18/33 (54%) | 11/19 (58%) | 3/8 (38%) | 2/3 (66%) | 2/10 (20%) | 13/21 (62%) |
| D11S912 | 23/49 (47%) | 18/37 (49%) | 12/21 (57%) | 3/7 (43%) | 2/4 (50%) | 3/13 (23%) | 14/22 (64%) |

| LOCUS | MOD/WELL DIFF | POORLY DIFF | DEBULK | NO DEBULK | ALIVE | DEAD | BENIGN/LMP |
|---|---|---|---|---|---|---|---|
| D11S922 | 5/13 (38%) | 8/20 (40%) | 10/26 (38%) | 4/8 (50%) | 5/17 (29%) | 10/18 (56% | 3/5 (60%) |
| D11S569 | 4/12 (33%) | 10/16 (62%) | 9/20 (45%) | 5/9 (56%) | 5/13 (38%) | 9/16 (56%) | 1/8 (12.5%) |
| D11S929 | 5/15 (33%) | 3/18 (17%) | 6/26 (23%) | 3/11 (27%) | 4/16 (25%) | 5/21 (24%) | 2/5 (40%) |
| D11S935 | 6/15 (40%) | 5/20 (25%) | 11/28 (39%) | 2/10 (20%) | 5/19 (26%) | 8/20 (40%) | 1/6 (17%) |
| D11S905 | 7/12 (58%) | 8/18 (44%) | 13/24 (54%) | 2/8 (25%) | 9/16 (56%) | 6/16 (38%) | 3/7 (43%) |
| D11S873 | 5/7 (71%) | 3/12 (25%) | 6/13 (46%) | 3/8 (37.5) | 4/7 (57%) | 5/14 (36%) | 1/2 (50%) |
| D11S35 | 4/13 (31%) | 7/21 (33%) | 11/25 (44%) | 4/9 (44%) | 6/17 (35%) | 7/21 (33%) | 1/4 (25%) |
| D11S897 | 4/13 (31%) | 4/16 (25%) | 5/21 (24%) | 4/10 (44%) | 2/15 (13%) | 7/16 (43%) | 2/5 (40%) |
| D11S925 | 5/11 (45%) | 10/19 (52%) | 14/22 (64%) | 4/9 (44%) | 6/15 (40%) | 9/17 (53%) | 2/9 (33%) |
| D11S912 | 5/15 (33%) | 10/18 (56%) | 9/24 (38%) | 8/11 (73%) | 4/17 (27%) | 14/19 (74% | 1/7 (14%) |

*Values given are the number of cases with LOH in that subgroup/total number of informative cases in that subgroup (% LOH in brackets). Early FIGO= FIGO stage I+II. Advanced FIGO=FIGO stage III+IV. Alive/dead refers to this status at 2 years minimum follow-up. Debulk=complete debulking at primary operation. No debulk=incomplete debulking at operation. LMP=tumours of low malignant potential (borderline tumours)*

In summary, 52/60 of all ovarian tumours (87%), and 40/47 EOCs (adenocarcinomas excluding borderline malignancies) (85%) had evidence of LOH involving at least one locus on chromosome 11. Only

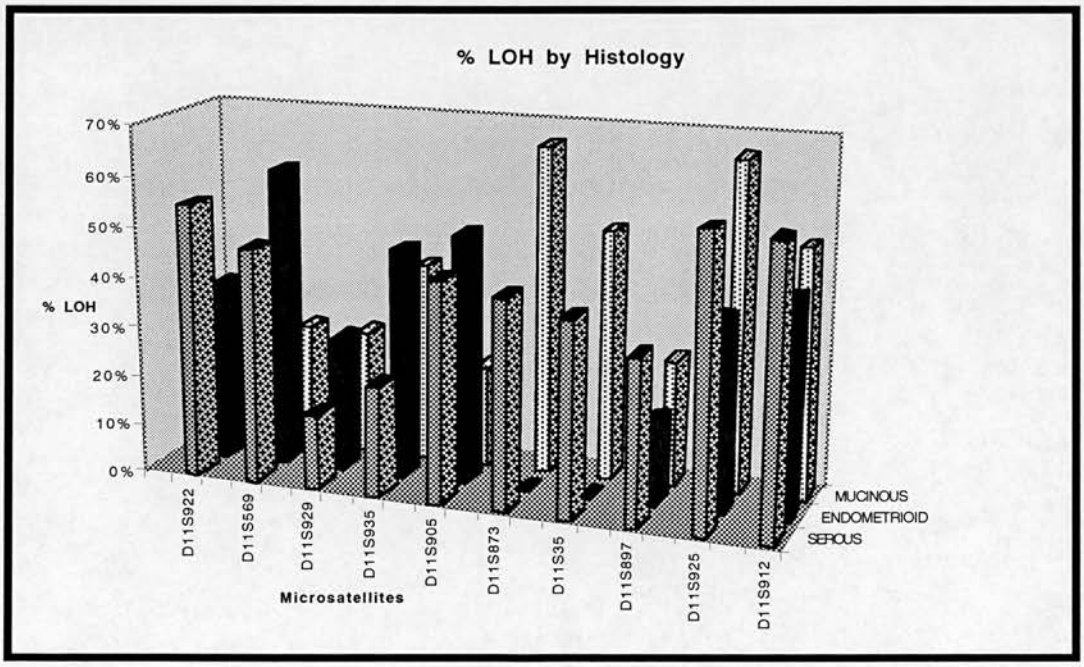
one EOC had LOH at all informative loci and 7 EOCs (15%) had no detectable LOH.

Serous, poorly differentiated and advanced stage EOCs had particularly high levels of LOH at both the 11p and 11q sub-telomeric regions. Conversely, EOCs which are early stage or moderately/well differentiated appear to have high levels of LOH at the 11q14.3-q22 region. The data from Table 3.3 above is summarised graphically below.

### 3.1.1 Allele imbalance and histology

No significant difference was seen at any locus, comparing serous EOCs with other histologies (Figure 3.2). However, of 9 informative endometrioid tumours, none had LOH at D11S35, and comparing this group with other histologies a trend towards significance was observed for LOH of this marker with non-endometrioid histology (p=0.04).

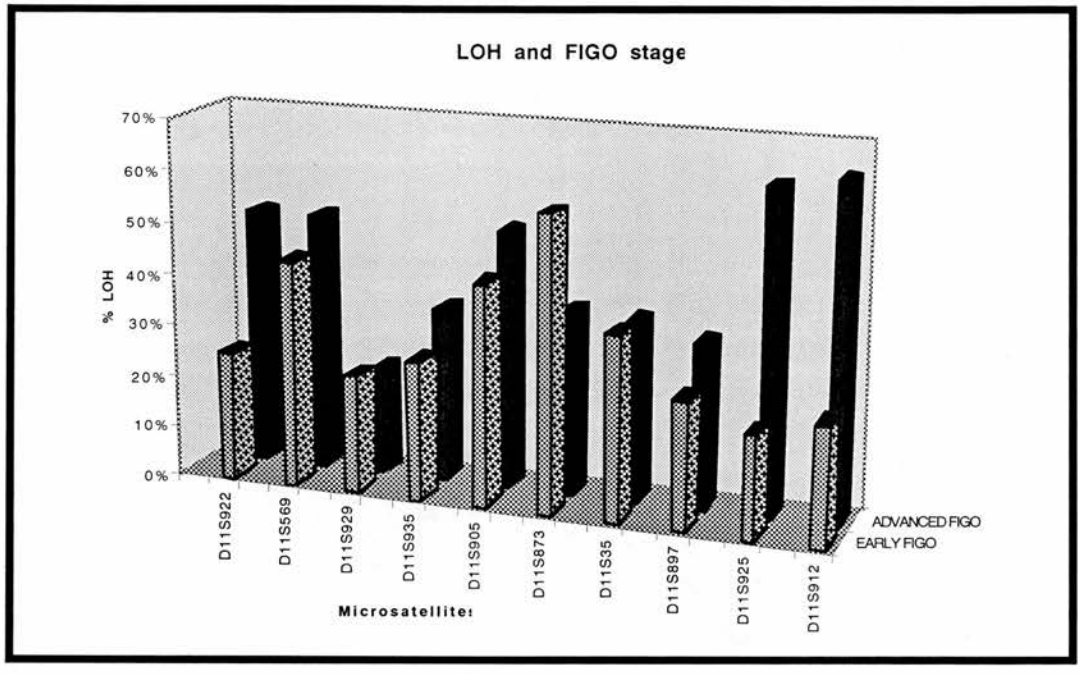
**Figure 3.2**




### 3.1.2 Allele imbalance and FIGO stage

The only observed trend towards significance was for the association of LOH at D11S912 with FIGO stage III/IV EOCs (p=0.035, see Figure 3.3).

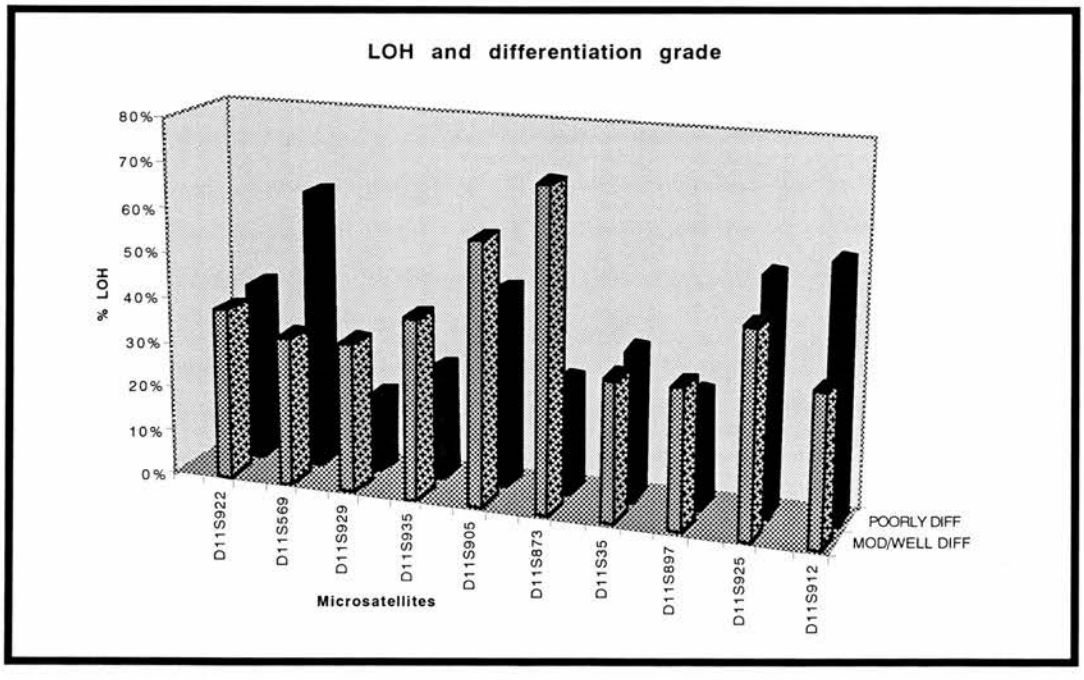
Figure 3.3




### 3.1.3 Allele imbalance and differentiation grade

The only apparent trend towards significance was between D11S873 LOH and well/moderately differentiated tumours (p=0.07).

Figure 3.4



### 3.1.4 Allele imbalance in other ovarian tumour types

The non-EOC-tumour numbers were too small for statistically valid conclusions. We considered benign and borderline (low malignant potential, LMP) tumours together. Only 1/8 benign/borderline tumours had LOH at D11S569 (11p15.3) and 1/7 had loss at D11S912 (11q24.1). However, 3/5 benign/borderline tumours had LOH at D11S922 (11p15.5). Both the mixed mesodermal tumours had LOH at both 11p15.5-p15.3 and 11q23-qter. One granulosa cell tumour had LOH at all loci on 11p suggesting whole arm loss. The ovarian teratoma in our series was hemizygous at all 9 informative loci and this is compatible with the usual description of these tumours as being parthenogenetic.

### 3.1.5 Microsatellite Instability

Microsatellite instability (MSI) was noted in only 6.4% of EOCs (3/47). 2/2 granulosa cell tumours had evidence of MSI and one of these tumours had evidence of instability at 3 loci. There were no cases of MSI in 5 borderline, 3 benign, and 2 mixed mesodermal tumours.

### 3.1.6 Analysis of consensus regions of allele loss in ovarian cancer

*11p Loss of Heterozygosity*

LOH was observed for at least 1 short-arm locus in 77% (46/60) of all informative tumours including 72% of EOCs (34/47).

For all ovarian tumours, high levels of LOH (>40%) were found for three loci (see table 3.3): D11S922 at 11p15.5 in 24/47 informative tumours (51%) and 16/36 EOCs (44%); D11S569 at 11p15.3 in 23/43 informative tumours (54%) and 14/30 EOCs (47%); and at D11S905 at 11p13-12 in 21/45 informative tumours (47%) and 15/33 EOCs (45%).

When considering only those tumours that were informative at both loci telomeric to 11p15.3 (D11S569 and D11S922) the rate of 11p sub-telomeric LOH was 16/24 (67%).

The lowest frequencies of allele loss on 11p were detected at D11S929 (11p14.1) with only 28% LOH in ovarian tumours and 24% LOH in EOCs.

*11q Loss of Heterozygosity*

11q LOH was observed for at least 1 locus in 65% (39/60) of all informative tumours including 66% of EOC's(31/47)

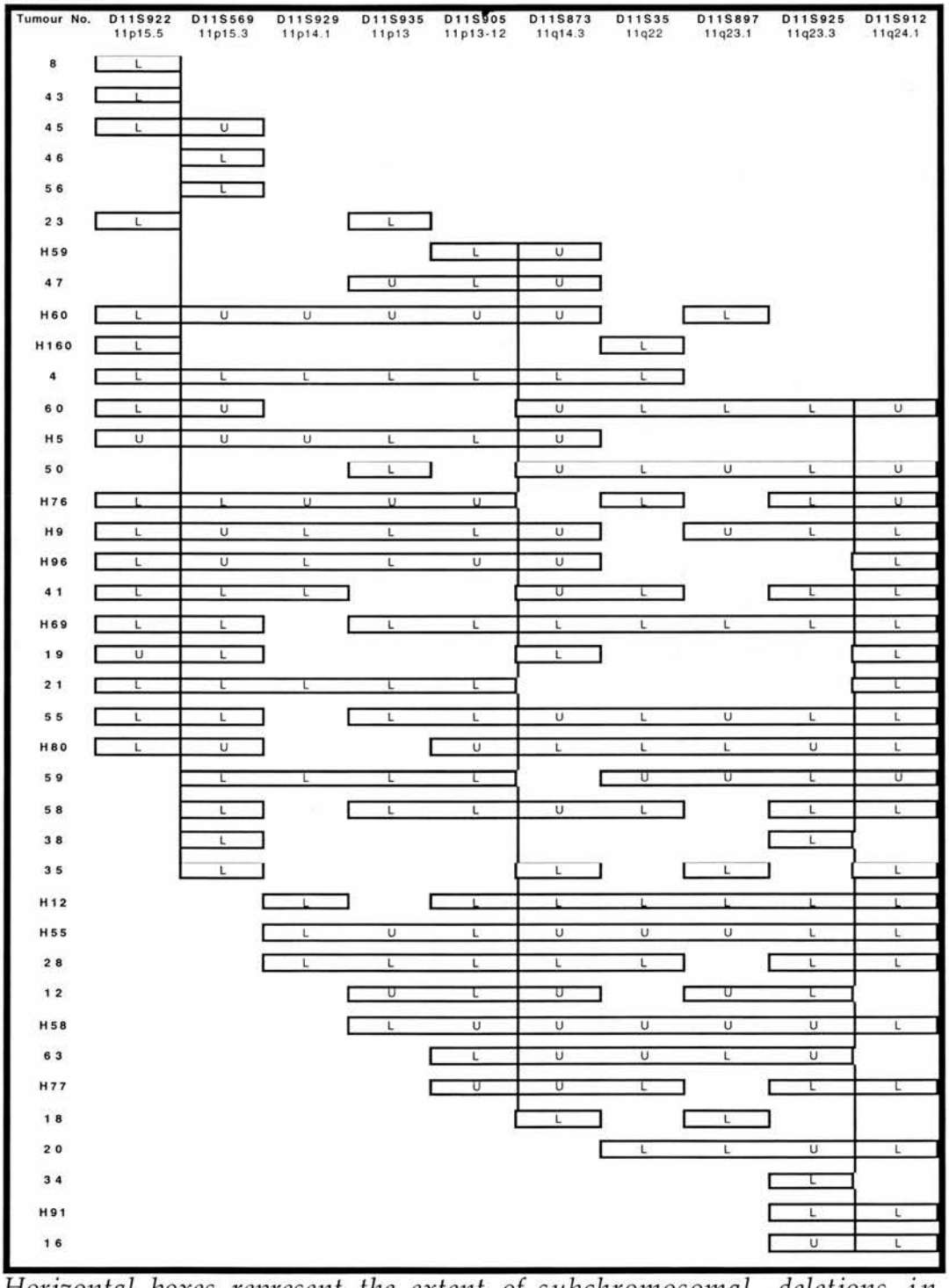
For all ovarian tumours, high levels of LOH were seen at three loci (see table 3.3): D11S873 at 11q14.3 in 11/25 informative tumours (44%) and 9/22 EOCs (41%); D11S925 at 11q23.3 in 24/45 informative tumours (53%) and 18/33 EOCs (54%); and D11S912 at 11q24.1 in 23/49 informative tumours (47%) and 18/37 EOCs (49%).

When considering only those EOCs that were informative at both loci telomeric to 11q23.3 (D11S925 and D11S912) the LOH rate was 18/27 (67%).

The lowest frequencies of allele loss were detected at D11S897 (11q23.1) with only 32% LOH in ovarian tumours and 28% LOH in EOCs.

Figure 3.5 shows those tumours that demonstrated partial loss on chromosome 11. Deletions are shown in shaded bars and are limited by the next heterozygous locus. In cases where a locus with allele loss is separated from a locus that remains heterozygous by an uninformative locus, that uninformative locus is included within the shaded bars as part of the deletion (since this region could be deleted). 8 tumours had 11p only loss, 6 tumours had 11q only loss and 25 tumours had partial loss of both arms. This type of analysis suggests 3 shortest regions of overlap (SRO's) corresponding to 3 consensus regions of deletion/allele imbalance at 11p15.5-15.3, 11q23.3-qter and 11p12-q22.

**Figure 3.5 Partial deletions reveal subchromosomal consensus regions of LOH**




*Horizontal boxes represent the extent of subchromosomal deletions in EOCs. Black vertical lines represent approximate positions of the shortest regions of overlap (SROs); three such regions are present. L= constitutive heterozygosity with LOH in tumour material. U=uninformative (constitutively homozygous). Constitutively heterozygous pairs with no LOH are left blank*

### 3.1.7 Statistical analysis

Fisher's exact test was used to analyse the relationship for allele imbalance between specific loci and also the relationship between imbalance for specific loci and clinicopathological parameters for EOCs.

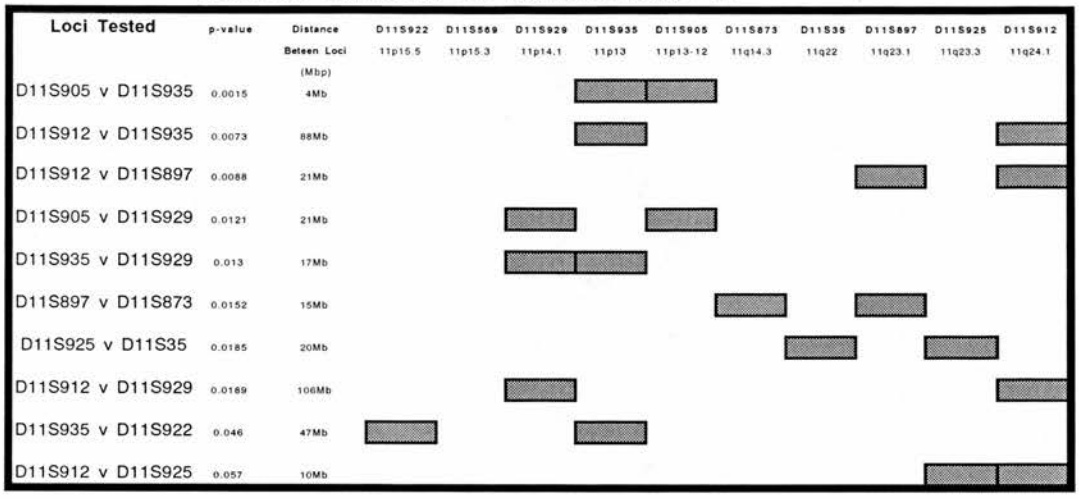
### *3.1.8* Relationship of allele imbalance between different loci

The three regions of deletion determined from figure 3.5 were analysed for significant relationships.

No relationship was noted between LOH at 11p sub-telomere region (D11S922 and D11S569 at 11p15.5-15.3) and 11q sub-telomere region(D11S925 and D11S912 at 11q23.3-24.1) (p= 0.5). Similarly, no relationship was seen between LOH at 11p12-q14.3 (D11S905 and D11S873), and 11p sub-telomere (p=1.0). However, a significant relationship exists for LOH at the 11p12-q14.3 region and the 11q sub-telomeric region (p=0.0025) suggesting that losses at these two regions co-segregate. Alternatively they could be part of a single deletion group although figure 3.5 suggests that this is not the case.

Fisher's exact test was used to test allele imbalance between all possible combinations of loci and the findings are presented in Table 3.4. For loci adjacent or close to each other, significant and borderline significant findings are likely to reflect association simply as part of substantial sub-chromosome deletions which may include a tumour suppressor gene.

**Table 3.4 Fisher's exact test analysis of co-loss between various markers on chromosome 11**




For loci distant from each other, note that D11S912/D11S935 LOH showed a significant statistical relationship (p=0.0073) and additionally, the relationship for D11S935/D11S922 was of borderline significance (p=0.046) suggesting the possibility that these loci harbour genes which may be inactivated co-operatively.

### 3.1.9 Relationships between LOH and clinicopathological parameters

Figures 3.2, 3.3 and 3.4 suggested differences in LOH rates between different clinicopathological parameters. Table 3.5 shows Fisher's test p-values with significance trends for clinicopathological parameters at the loci tested.

**Table 3.5 Significant associations between LOH and clinicopathological factors**

| LOH at | LOCUS | ASSOCIATION | p-VALUE |
|---|---|---|---|
| **D11S912** | 11q24.1 | Dead (vs alive) with min. 24 months follow-up | 0.0067 |
| **D11S912** | 11q24.1 | Dead (vs alive) at 24 month follow-up point | 0.067 |
| **D11S912** | 11q24.1 | Late FIGO stage (vs Early FIGO) at presentation | 0.035 |
| **D11S912** | 11q24.1 | Non-debulked(vs completely debulked) post initial surgery | 0.075 |
| **D11S35** | 11q22 | Non-Endometrioid (vs Endometrioid) histology | 0.04 |
| **D11S873** | 11q14.3 | Well/moderately (vs poorly) differentiated tumours | 0.07 |

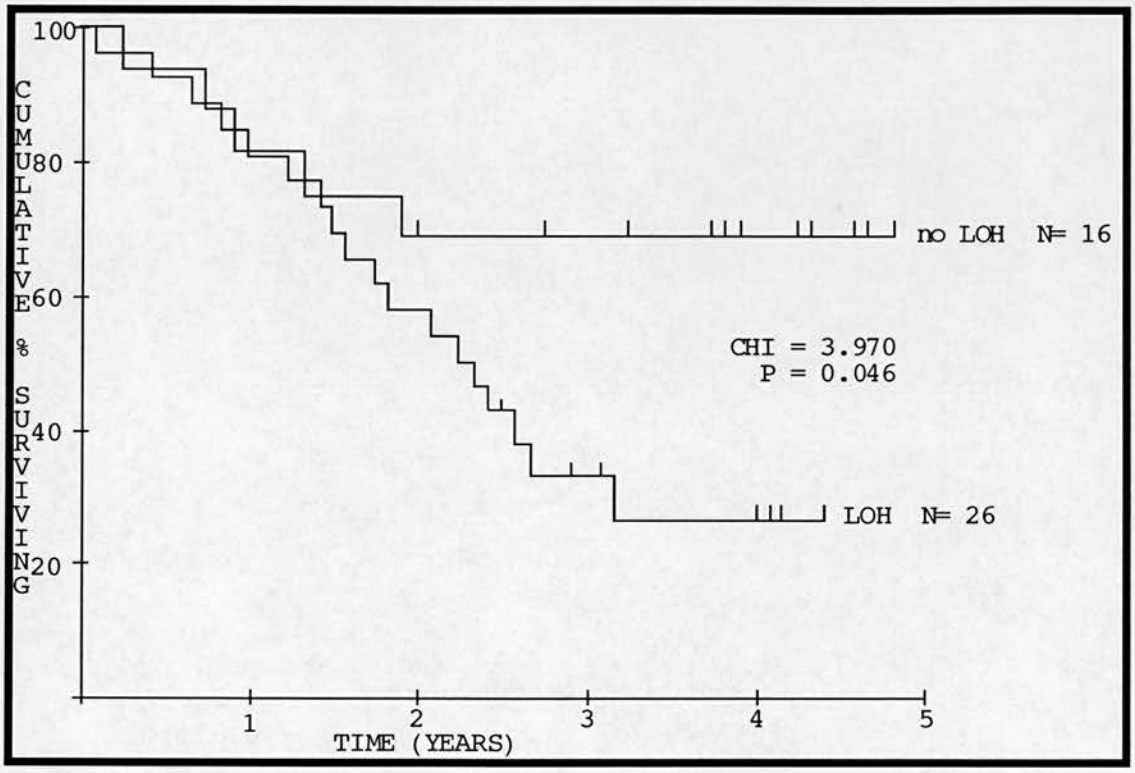
### 3.1.10 Survival analysis

D11S912 loss of heterozygosity at 11q23.3-q24.1 in primary tumours at diagnosis was associated with adverse survival of patients with adenocarcinoma, (p=0.0067) with minimum follow-up of 24 months.

*Kaplan-Meier survival analysis*

Sub-telomeric 11q LOH (D11S925 and D11S912) was significantly associated with adverse survival for patients with ovarian adenocarcinoma (Figure 3.6).

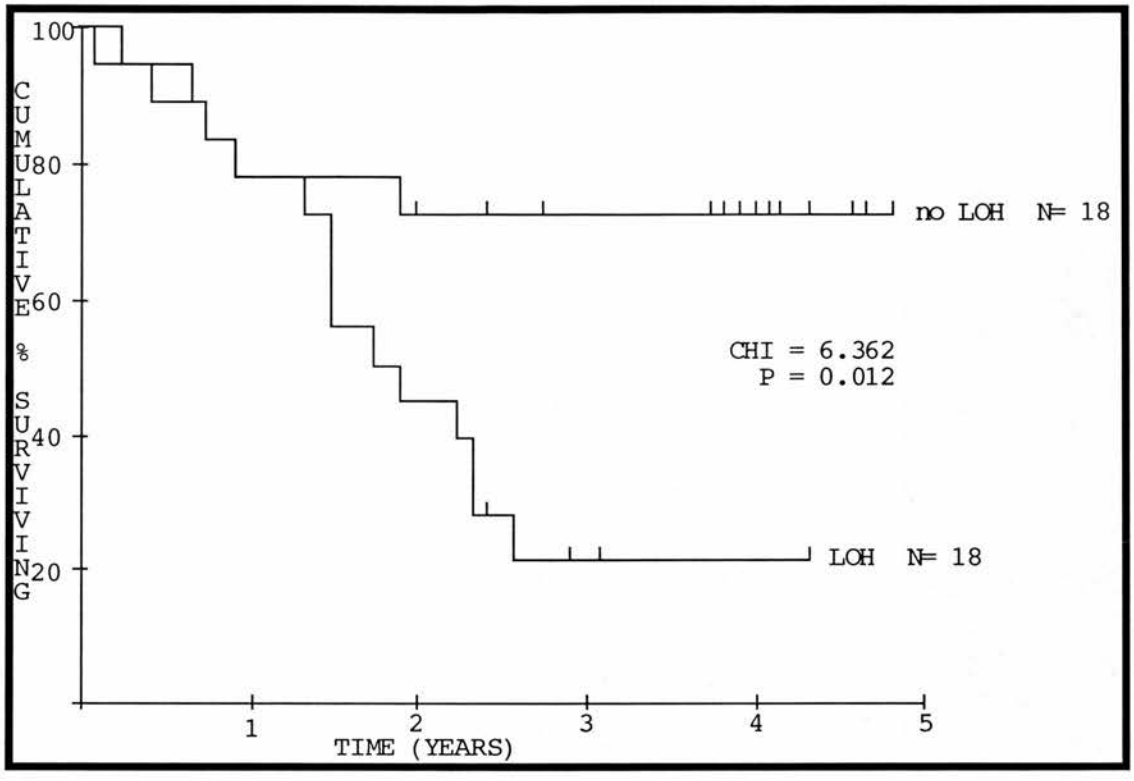
**Figure 3.6 Survival of ovarian cancer patients by LOH status**




*Kaplan-Meier survival curve with log-rank analysis for sub-telomeric 11q LOH status at presentation (found in primary tumour)*

Significance was increased when D11S912 LOH was considered alone (Figure 3.7). Actuarial survival of those without LOH shows 70% survival at 4 years versus 20% for patients who had lost a D11S912 allele in their primary tumour at diagnosis.

**Figure 3.7 Survival of ovarian cancer patients by LOH status of D11S912**




*Kaplan-Meier survival curve with log-rank analysis D11S912 LOH status at presentation.*

## 3.2 Integration of chromosome 11 and 17 LOH data from patients with ovarian cancer: statistical analysis

### 3.2.1 Rationale

The initial chromosome 11 study (Gabra et al, 1995a) was performed in Professor Michael Steel's laboratory using a cohort which included many blood tumour pairs collected by Dr Diana Eccles who was a research fellow in Professor Steel's laboratory. Dr Eccles and colleagues had extensively analysed chromosome 17p and a chromosome 17q (Eccles et al, 1990) marker in this series and Ms Lorna Gruber (now Mrs Lorna Taylor) had continued to examine 17q for LOH in some detail. This allowed me to analyse the tumours for chromosome 11/17 LOH associations and relative strength of associations of LOH with other parameters. The patient cohort examined was the same cohort as

above although subgroup statistical analysis was limited in that many tumours were collected by me and therefore did not have chromosome 17p analysed in detail, although Lorna Gruber did perform 17q LOH analysis on these tumours.

Figure 3.8 shows an overview of the markers used for this part of the statistical analysis.

**Figure 3.8: Chromosomal location of polymorphic microsatellites used for the chromosome 11/17 statistical analysis**

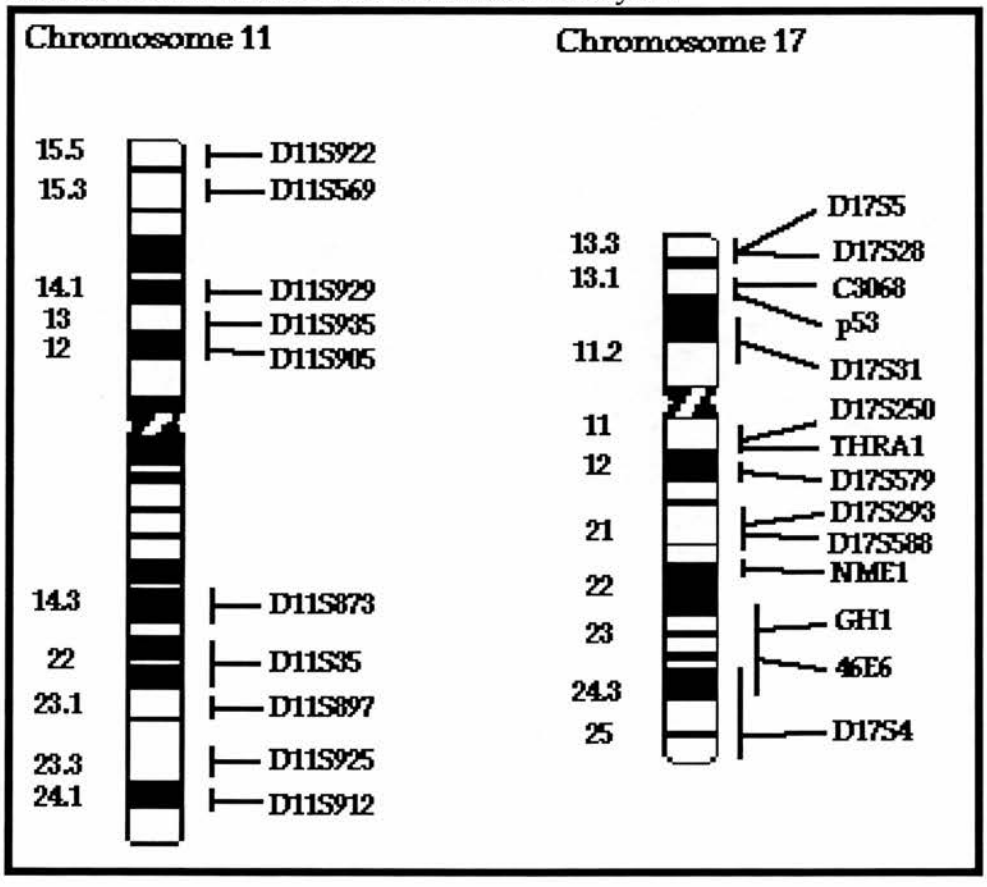



Table 3.6 shows the frequency of LOH at the loci tested and their location (The chromosome 11 data mentioned above is reiterated, and Diana Eccles' chromosome 17p and D17S4 data is also mentioned (with permission) in order to give a contextual overview).

Overall, chromosome 17 LOH was generally higher than chromosome 11 LOH. Rates were lowest at GH1 on chromosome 17q and D11S929 on chromosome 11p.

Table 3.6 **Locus name, position and LOH rate for markers used in the chromosome 11/17 analysis**

| | CHROMOSOME 17 | | | | | CHROMOSOME 11 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Marker | Location | LOH | informative | %LOH | Marker | Location | LOH | informatives | %LOH |
| p arm | D17S5 | 17p13.3 | 9 | 17 | 53% | D11S922 | 11p15.5 | 16 | 36 | 44% |
| | D17S28 | 17p13.3 | 9 | 19 | 47% | D11S569 | 11p15.3 | 14 | 30 | 47% |
| | C3068 | 17p13.3-p13.1 | 9 | 19 | 47% | D11S929 | 11p14.1 | 9 | 38 | 24% |
| | p53 | 17p13.1 | 11 | 20 | 55% | D11S935 | 11p13 | 13 | 39 | 33% |
| | D17S31 | 17p13.1-p11.2 | 5 | 13 | 38% | D11S905 | 11p13-p12 | 15 | 33 | 45% |
| q arm | D17S250 | 17q11-q12 | 15 | 29 | 52% | D11S873 | 11q14.3 | 9 | 22 | 41% |
| | THRA1 | 17q11.2-q12 | 17 | 28 | 61% | D11S35 | 11q22 | 14 | 39 | 36% |
| | D17S579 | 17q12-q21 | 18 | 35 | 51% | D11S897 | 11q23.1 | 9 | 32 | 28% |
| | D17S293 | 17q21 | 14 | 32 | 44% | D11S925 | 11q23.3 | 18 | 33 | 54% |
| | D17S588 | 17q21 | 18 | 33 | 55% | D11S912 | 11q24.1 | 18 | 37 | 49% |
| | NME 1 | 17q21-q22 | 25 | 38 | 66% | | | | | |
| | GH1 | 17q22-q24 | 9 | 33 | 27% | | | | | |
| | 46 E 6 | 17q22-q24.3 | 23 | 41 | 56% | | | | | |
| | D17S4 | 17q23-q25.3 | 13 | 19 | 68% | | | | | |

### 3.2.2 Chromosome 17 and 11 regions of co-ordinate loss

The polymorphic marker LOH data were initially used for a "whole arm" analysis to test if some of the chromosome arms were lost together more frequently than predicted by chance (Table 3.7). In this analysis, co-loss of 17p and 17q was noted with a high degree of significance (p=0.0003). Co-loss of 17q and 11q also showed a trend to significance (p=0.035) and this trend became more significant when the telomeric half of 11q was considered (p=0.0175).

**Table 3.7 Chr 11/17 LOH whole arm associations**

| Whole arm co-losses | p-value | n |
|---|---|---|
| 17p vs 17q | 0.0003 | 37 |
| 17p vs 11p | 1 | 34 |
| 17q vs 11q | 0.0351 | 41 |
| 17p vs 11q | 0.119 | 32 |
| 17q vs 11p | 0.624 | 43 |
| 11p vs 11q | 0.651 | 41 |
| 17q vs 11q23-qter | 0.0175 | 40 |

Individual loci were then examined for evidence of significant co-ordinate loss (Table 3.8). As demonstrated above (Table 3.4), adjacent loci demonstrating significant co-loss were ignored since this might have been due to contiguous deletions. Within the confines of this assumption, NME1 (17q21) and D11S922 (11p15.5) were lost together very significantly (p=0.0067) as were D11S912 (11q24.1) and D11S935 (11p13) (p=0.0073). Co-ordinate losses of p53/D17S4 (17p13/17q25) and D11S912/D11S929 (11q24/11p14) both occurred at borderline significance. The only individual loci in this study demonstrating 17q/11q co-loss were Thra 1 and D11S35 at borderline significance (p=0.049).

Table 3.8 **Statistical associations between LOH at defined polymorphic loci**

| Co-Losses | Location | number informative | p-value |
|---|---|---|---|
| NME1 and D11S922 | 17q21-22 and 11p15.5 | 30 | 0.0067 |
| D11S912 and D11S935 | 11q24.1 and 11p13 | 32 | 0.0073 |
| p53 and D17S4 | 17p13.1 and 17q23-25 | 12 | 0.018 |
| D11S912 and D11S929 | 11q24.1 and 11p14.1 | 34 | 0.019 |
| THRA-1 and D11S35 | 17q11-12 and 11q22 | 18 | 0.049 |

### 3.2.3 LOH and associations with clinicopathological factors.

Fisher's test was then used to examine the relationship between whole arm or individual locus LOH with clinicopathological prognostic factors.

The only significant whole arm LOH association (Table 3.9) was between 17p LOH and serous type histology (p=0.0052). There was also a trend towards significance for 17p LOH with poorly differentiated tumours (p=0.025) and advanced FIGO stage (p=0.031), but no association with survival (p=0.171). The only other "whole arm" trend to significance was found for 11q LOH with adverse survival (p=0.031).

**Table 3.9 Statistical associations between chromosome arm loss and clinicopathological features of ovarian cancer**

| Prognostic parameter asociated | chromosome arm associated | number informative | Fisher's p-value |
|---|---|---|---|
| Serous histology | 17p | 37 | 0.0052 |
| | 17q | 47 | 0.123 |
| | 11p | 43 | 1 |
| | 11q | 41 | 0.306 |
| Poorly differentiated tumour | 17p | 34 | 0.025 |
| | 17q | 42 | 0.099 |
| | 11p | 38 | 0.709 |
| | 11q | 36 | 0.483 |
| Advanced FIGO stage | 17p | 35 | 0.031 |
| | 17q | 44 | 0.434 |
| | 11p | 40 | 0.689 |
| | 11q | 38 | 0.061 |
| Adverse survival outcome | 17p | 36 | 0.171 |
| | 17q | 46 | 0.117 |
| | 11p | 42 | 0.708 |
| | 11q | 40 | 0.031 |

Individual loci were then examined for an association between LOH and prognostic clinicopathological factors (Table 3.10). In this part of the analysis, LOH of individual 17p loci always occurred in concert, and so 17p loss was considered as a single entity. D17S4 (17q25) LOH was highly significantly associated with serous histology (p=0.0007) (loss of D17S4 co-incided significantly with loss of 17p, associated with serous histology). D17S588 LOH (17q21) was significantly associated with advanced FIGO stage (p=0.0075). LOH at two loci were significantly associated with adverse survival; D11S912 (11q24; p=0.0067) and Thra 1 (17q11-12; p=0.0076). Other trends towards

significance are shown in table 7 including two associations of LOH which deserve further comment: D11S35 LOH was associated with non-endometrioid histology (p=0.04), interesting because of the proximity of the progesterone receptor to this locus (35); and D11S873 LOH associated with moderate/well differentiated tumours at borderline significance (p=0.07).

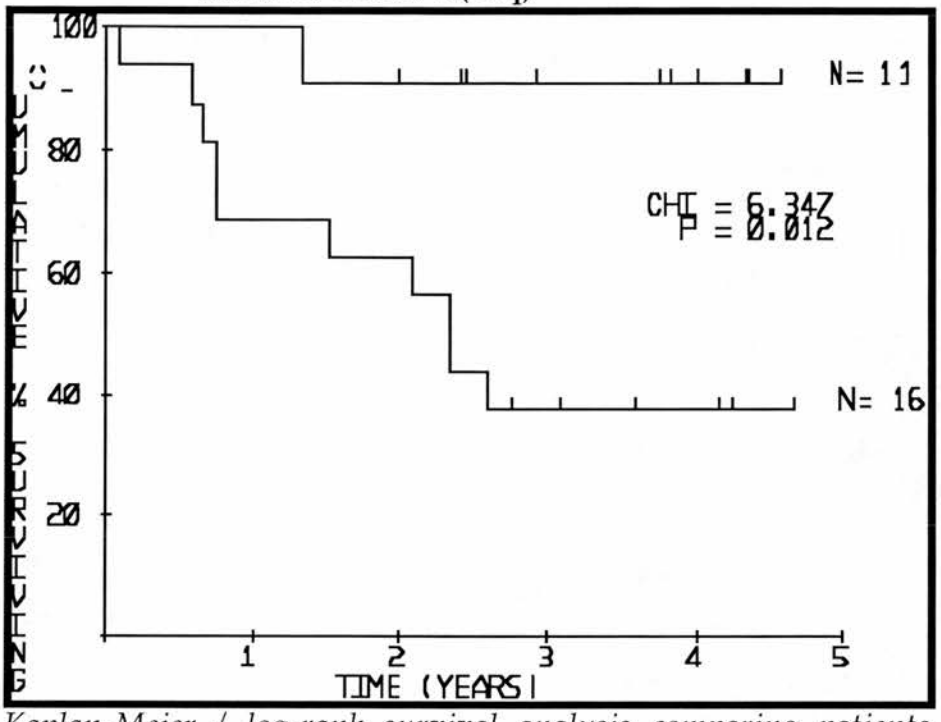
**Table 3.10 Statistical association between defined polymorphic loci and clinicopathological features of ovarian cancer**

| Prognostic parameter associated with LOH | microsatellite marker associated | Location | number informative | significance of statistical association |
|---|---|---|---|---|
| Histology: | | | | |
| serous | D17S4 | 17q23-25.3 | 18 | 0.0007 |
| | 17p | | 37 | 0.0052 |
| | D17S588 | 17q21 | 33 | 0.038 |
| non-endometrioid | D11S35 | 11q22 | 27 | 0.04 |
| Differentiation: | | | | |
| poor | D17S4 | 17q23-25.3 | 18 | 0.022 |
| | D17S579 | 17q12-21 | 31 | 0.023 |
| | 17p | | 34 | 0.025 |
| | THRA-1 | 17q11.2-12 | 24 | 0.03 |
| moderate/well | D11S873 | 11q14.3 | 19 | 0.07 |
| Advanced FIGO stage | D17S588 | 17q21 | 31 | 0.0075 |
| | D11S912 | 11q24.1 | 35 | 0.035 |
| Adverse survival outcome | D11S912 | 11q24.1 | 36 | 0.0067 |
| | THRA-1 | 17q11.2-12 | 27 | 0.0076 |
| | NME1 | 17q21-22 | 37 | 0.047 |

### 3.2.4 Survival analysis

In addition to the findings for 11q LOH in association with adverse survival(see figures 3.6 and 3.7 above), Kaplan-Meier / log-rank analysis confirmed Fisher's exact test findings with respect to Thra 1 on 17q, with a significant actuarial survival disadvantage for those who had LOH at this locus at presentation (Figure 3.9). No other survival associations were revealed in this analysis.

**Figure 3.9 Survival of ovarian cancer patients by LOH status at THRA-1 (17q)**




*Kaplan-Meier / log-rank survival analysis comparing patients with or without LOH at THRA-1*

### 3.3 LOH close to the progesterone receptor (PgR) locus in ovarian cancer

#### 3.3.1 Rationale

As mentioned in the preceding section, the progesterone receptor (PgR) is of interest in understanding the endocrine regulation of ovarian cancer, itself a component of growth regulation, and PgR protein is implicated in growth suppression and differentiation of breast and ovarian cancer. In this study we used a polymorphic microsatellite lying close to the PgR gene to determine allele loss in ovarian cancer. At the time of the study no intragenic microsatellite with high heterozygosity was available for the PgR gene, RFLP analysis for the PgR gene being even less informative. D11S35 was selected because it was the closest microsatellite to the PgR gene (with an indistinguishable map location at 0.41 and 0 cM separation) and had an acceptably high heterozygosity rate (Litt et al, 1990; Litt et al, 1993).

Subsequent to completing the study a high resolution physical map of chromosome 11 suggested that it lay slightly (3 centirays, approximately 150 kb) telomeric to the PgR gene (James et al, 1994).
PgR and ER content of these tumours was then measured and the relationship between LOH at the PgR locus and tumoural steroid receptor content was assessed. Two other incidental polymorphic microsatellites were randomly selected to demonstrate specificity of the correlation: D11S935 (11p13) (Weissenbach et al, 1992), and NM23 (17q22) (Hall et al, 1992)

### 3.3.2 LOH analysis

Of 47 EOC patients, 38 were informative (constitutively heterozygous) at D11S35 (72%). 14 of these (37%) exhibited LOH at this locus and 24 retained heterozygosity. There was inadequate tissue for ER and PgR quantification in 7 of these informative specimens (2 with LOH and 5 heterozygous, no loss) and therefore the final allele loss rate in this group was 12/31 (39%). For D11S935 the final allele loss rate was 13/33 (39%) and for NM23 (17q22), the final allele loss rate was 19/31 (61%).
The sample contained 9 informative endometrioid ovarian cancers, none of which exhibited LOH at D11S35. Note that the initial chromosome 11 analysis identified a significant correlation between D11S35 retention of heterozygosity and endometrioid histology ($p=0.04$) in the 38 informative tumours. This data is summarised in Table 3.11 (next page).

**Table 3.11 Clinicopathologic parameters and hormone receptor content in relation to LOH status of the patient series**

| TUMOR | SURVIVAL (months) | ALIVE ? | STAGE (FIGO) | HISTOLOGY | GRADE | D11S35 11q22 | NM23 17q22 | D11S935 11p13 | PR fmol/mg | ER protein |
|---|---|---|---|---|---|---|---|---|---|---|
| G3 | 9 | | 3 | SEROUS | P.D. | □ | □ | □ | 159 | 29 |
| G4 | 58 | A | 1 C | CLEAR CELL | M.D. | ■ | U | ■ | 2 | 5 |
| G8 | 56 | A | 2 | SEROUS | P.D. | □ | ■ | U | 100 | 12 |
| G11 | 27 | | 3 | SEROUS | M.D. | U | U | ■ | 100 | 12 |
| G12 | 5 | | 4 | SEROUS | P.D. | □ | ■ | U | 1 | 1 |
| G16 | 21 | | 3 | MUCINOUS | NS | □ | ■ | □ | 1 | 1 |
| G17 | 52 | A | 3 | SEROUS | M.D. | U | U | □ | 2 | 3 |
| G18 | 11 | | 1 A | MUCINOUS | M.D. | □ | ■ | □ | 29 | 8 |
| G20 | 23 | | 3 | SEROUS | P.D. | ■ | □ | □ | 4 | 229 |
| G23 | 10 | | 3 | ENDOMETRIOID | M.D. | □ | U | ■ | 21 | 9 |
| G24 | 51 | A | 3 | SEROUS | P.D. | □ | ■ | □ | 2 | 160 |
| G26 | 7 | | 3 | SEROUS | P.D. | □ | U | □ | 38 | 39 |
| G28 | 52 | A | 1 A | MUCINOUS | M.D. | ■ | □ | ■ | 7 | 6 |
| G30 | 23 | | 1 A | ENDOMETRIOID | P.D. | □ | U | □ | 12 | 12 |
| G34 | 49 | A | 3 B | SEROUS | P.D. | □ | ■ | □ | 187 | 163 |
| G35 | 1 | | 3 | SEROUS | P.D. | □ | U | □ | 6 | 12 |
| G38 | 48 | A | 1 A | ENDOMETRIOID | M.D. | □ | □ | □ | 139 | 161 |
| G42 | 47 | A | 1 B | ENDOMETRIOID | P.D. | □ | ■ | □ | 9 | 10 |
| G46 | 46 | A | 1 A | MUCINOUS | W.D. | U | □ | □ | 1144 | 315 |
| G50 | 48 | A | 3 | MUCINOUS | NS | ■ | □ | ■ | 11 | 10 |
| G55 | 11 | | 4 | SEROUS | P.D. | ■ | ■ | ■ | 3 | 10 |
| G56 | 43 | A | 1 A | ENDOMETRIOID | P.D. | □ | ■ | □ | 6 | 11 |
| G58 | 18 | | 3 | SEROUS | P.D. | ■ | ■ | ■ | 6 | 19 |
| G59 | 38 | | 3 | ENDOMETRIOID | P.D. | U | ■ | ■ | 74 | 109 |
| G60 | 30 | | 1 C | SEROUS | P.D. | ■ | ■ | □ | 11 | 40 |
| H5 | 38 | A | 1 C | ENDOMETRIOID | P.D. | U | □ | ■ | 8 | 18 |
| H9 | 37 | A | 3 | ENDOMETRIOID | M.D. | □ | □ | ■ | 130 | 59 |
| H12 | 35 | A | 1 C | CLEAR CELL | M.D. | ■ | □ | □ | 3 | 7 |
| H55 | 8 | | 3 | SEROUS | P.D. | U | □ | U | 10 | 8 |
| H58 | 31 | | 1 | ENDOMETRIOID | M.D. | U | ■ | ■ | 4 | 116 |
| H59 | 33 | A | 1 | ENDOMETRIOID | M.D. | □ | U | □ | 99 | 30 |
| H60 | 16 | | 3 | ENDOMETRIOID | W.D. | □ | ■ | U | 13 | 10 |
| H69 | 25 | | 3 | SEROUS | P.D. | ■ | ■ | ■ | 5 | 35 |
| H76 | 25 | | 3 | SEROUS | P.D. | ■ | ■ | U | 3 | 88 |
| H77 | 27 | | 3 | CLEAR CELL | M.D. | ■ | ■ | □ | 3 | 48 |
| H80 | 18 | | 3 | SEROUS | NS | ■ | ■ | □ | 4 | 46 |
| H91 | 28 | | 3 | SEROUS | P.D. | □ | □ | □ | 3 | 21 |
| H95 | 24 | A | 1 C | ENDOMETRIOID | P.D. | U | □ | □ | 42 | 21 |
| H96 | 9 | | ? | ENDOMETRIOID | NS | □ | ■ | ■ | 21 | 8 |

*Clinicopathologic parameters and LOH status of the patient series. A = alive at most recent analysis, PR = progesterone receptor, ER = oestrogen receptor, U = un-informative/homozygous, filled boxes= loss of heterozygosity, open boxes = heterozygous with no loss.*

### 3.3.3 PgR distribution and relationships between LOH and PgR

Sufficient material was available for ER and PgR measurement in 39 of the 47 tumours (see Table 3.11), and this estimation was performed in a blind fashion at a different institution (by R.A. Hawkins and A.L. Tesdale, Royal Infirmary of Edinburgh). A summary of PgR data distribution by locus and allele loss status is shown in Table 3.12.

The mean tumoural PgR concentration for the 12 patients with LOH at D11S35 was 5.2 fmol/mg with a median of 4 fmol/mg and a range of 2-

11 fmol/mg. The mean tumoural PgR concentration for the 19 heterozygous patients with no loss at D11S35 was 51.4 fmol/mg with a median of 21 fmol/mg, and a range of 1-187 fmol/mg. For D11S935 the median PgR of the LOH group was 8 fmol/mg (range 2-130 fmol/mg), for the no LOH group it was 7.5 fmol/mg (range 1-1144 fmol/mg). For NM23 the median for the LOH group was 6 fmol/mg (range 1-187 fmol/mg), and for the no LOH group 10.5 fmol/mg (3-1144 fmol/mg). The difference between the medians in the D11S35 LOH/no LOH groups was statistically significant with the Mann-Whitney U-test (p=0.014). These analyses were repeated for a chromosome 11 short arm locus, D11S935 at 11p13; and for NM23, a chromosome 17 long arm locus (17q22). Although large apparent differences were also noted (see above) for the LOH and heterozygous/no loss groups at these two loci, no statistically significant differences of mean and median PgR concentration by allele loss status were recorded.

**Table 3.12 Distribution of PgR data by allele loss status for three microsatellites**

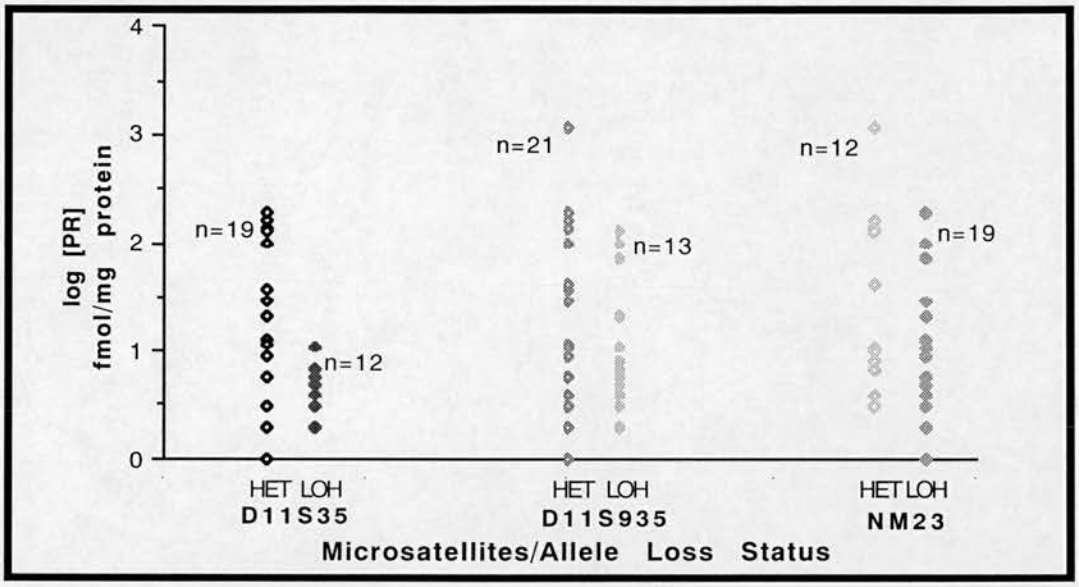
| | D11S35 | | | D11S935 | | | NME-1 | | |
|---|---|---|---|---|---|---|---|---|---|
| | LOH | 11q22 | HET | LOH | 11p13 | HET | LOH | 17q22 | HET |
| Number of patients | 12 | | 19 | 13 | | 20 | 19 | | 12 |
| Mean PgR | 5.2 | | 51 | 30 | | 93.2 | 25 | | 138 |
| Median PgR | 4 | | 21 | 8 | | 7.5 | 6 | | 10.5 |
| Standard Dev. | 3.1 | | 62 | 43 | | 254 | 47 | | 322 |
| Minimum | 2 | | 1 | 2 | | 1 | 1 | | 3 |
| Maximum | 11 | | 187 | 130 | | 1144 | 187 | | 1144 |
| Mann-Whitney U Statistic | 53 | | | 125 | | | 76.5 | | |
| 2-tailed p-value | 0.014 | | | 0.87 | | | 0.133 | | |

*associated non-parametric statistics compare the LOH with the heterozygous/no loss (HET) groups*

Plotting tumoural PgR concentration against D11S35 allele loss status as a log scatter plot shows the association of low/absent tumoural PgR

with D11S35 LOH, but this clear difference is not apparent for the other two markers (Figure 3.10).

**Figure 3.10 PgR content according to LOH status**

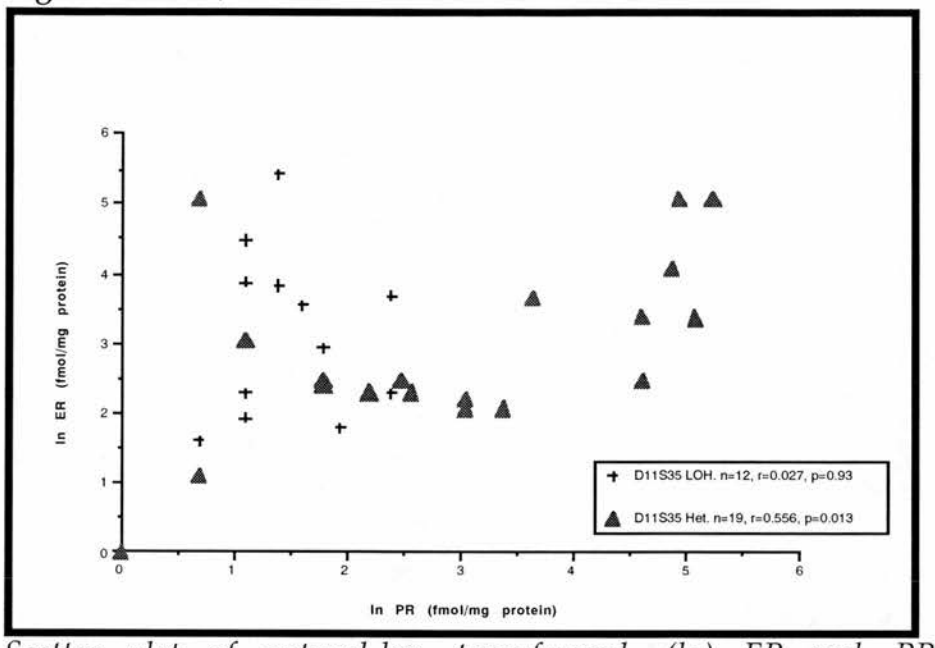



*Tumoural PgR content ($log_{10}$ transformed values) plotted against tumoural allele loss status for the three microsatellites used. HET=heterozygous at the locus without LOH.*

### 3.3.4 Relationship between ER and PgR

The whole D11S35 informative group was analysed for linear correlation between tumoural ER and PgR content by Spearman rank correlation. The ER and PgR values were natural-log transformed There was no significant correlation for the whole group. However, when patients with D11S35 LOH were removed from the analysis, there was a linear correlation between tumoural PgR and ER content (Spearman rank correlation, p=0.013). Figure 3.11 shows a scatter plot of D11S35 heterozygous/no loss group and LOH group in terms of their ER versus matched PgR contents.

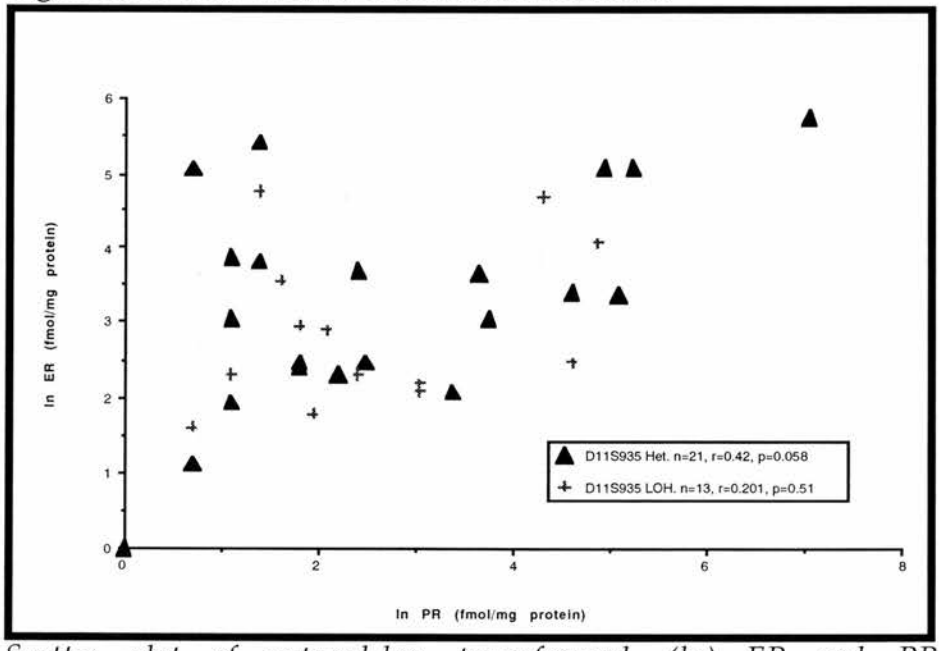
**Figure 3.11 ER/PR rank correlation: D11S35**




*Scatter plot of natural-log transformed (ln) ER and PR values comparing tumors with allele loss ( = LOH) and tumors that were heterozygous without LOH ( = Het). Data presented for D11S35 (11q22), (b) D11S935 (11p13), and (c) NM23.*

The analysis was repeated for D11S935 (figure 3.12)

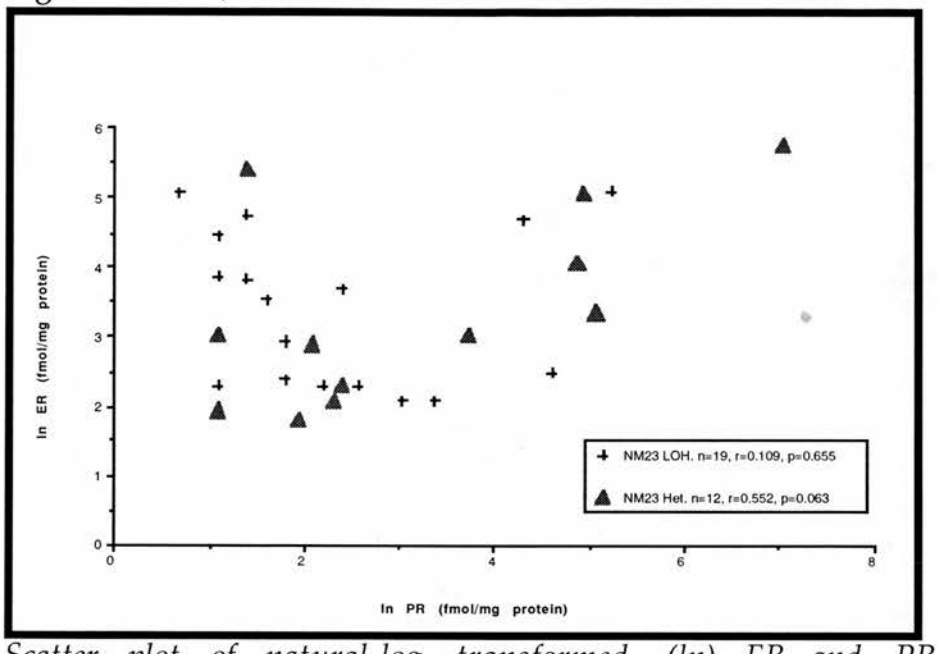
**Figure 3.12 ER/PR rank correlation: D11S935**




*Scatter plot of natural-log transformed (ln) ER and PR values comparing tumors with allele loss ( = LOH) and tumors that were heterozygous without LOH ( = Het). Data presented for D11S935.*

and NM23 (figure 3.13).

**Figure 3.13 ER/PR rank correlation: NM23**




*Scatter plot of natural-log transformed (ln) ER and PR values comparing tumors with allele loss ( = LOH) and tumors that were heterozygous without LOH ( = Het). Data presented for NM23.*

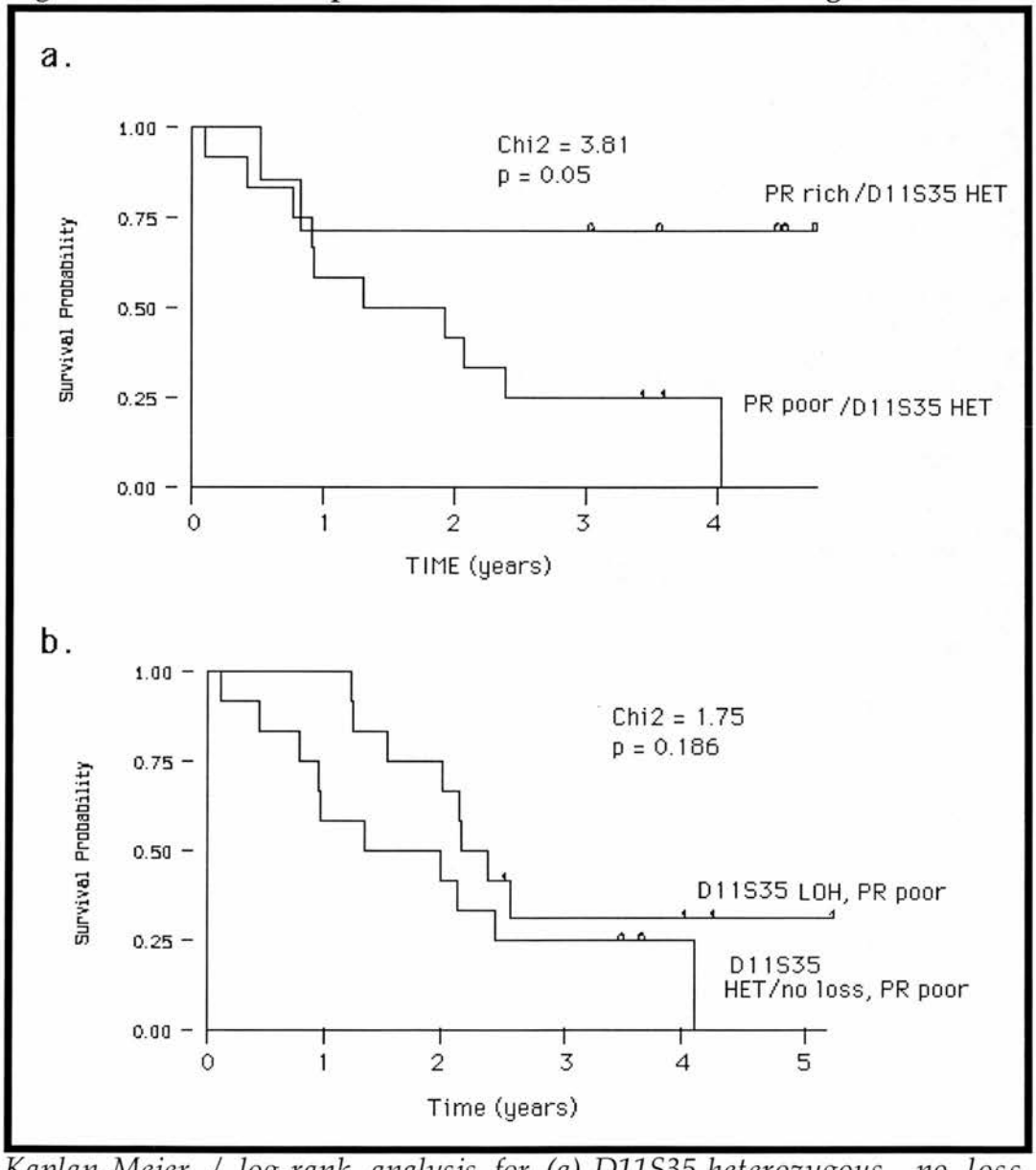
Although there was no significant correlation between ER and PgR content in either the heterozygous/no loss group or the LOH group for either of these latter markers, it is interesting that both had trends to significance in their respective heterozygous/no loss group, but these trends were not different between the D11S935 marker on 11p (p=0.058) and NM23 on 17q (p=0.063).

### 3.3.5 Survival analysis

Kaplan-Meier/log rank survival analysis for ER-rich versus ER-poor patients showed no significant difference between the two groups (data not shown). PgR-rich versus PgR-poor showed a non-significant trend in favour of the PgR-rich patients (p=0.08, data not shown). Comparing patients with tumour D11S35 LOH versus patients with D11S35 heterozygous/no loss tumours also showed no difference (data not shown). However, analysis of patients with D11S35 heterozygous/no loss tumours only showed a survival advantage for patients with PgR-rich (≥30 fmol/mg) tumours compared to those with PgR-poor (<30

fmol/mg) tumours (p=0.05, Figure 3.14a) and PgR poor patients did equally badly whether they had LOH at D11S35 or not (figure 3.14b).

**Figure 3.14 Relationship of D11S35 allele loss status and PgR status**

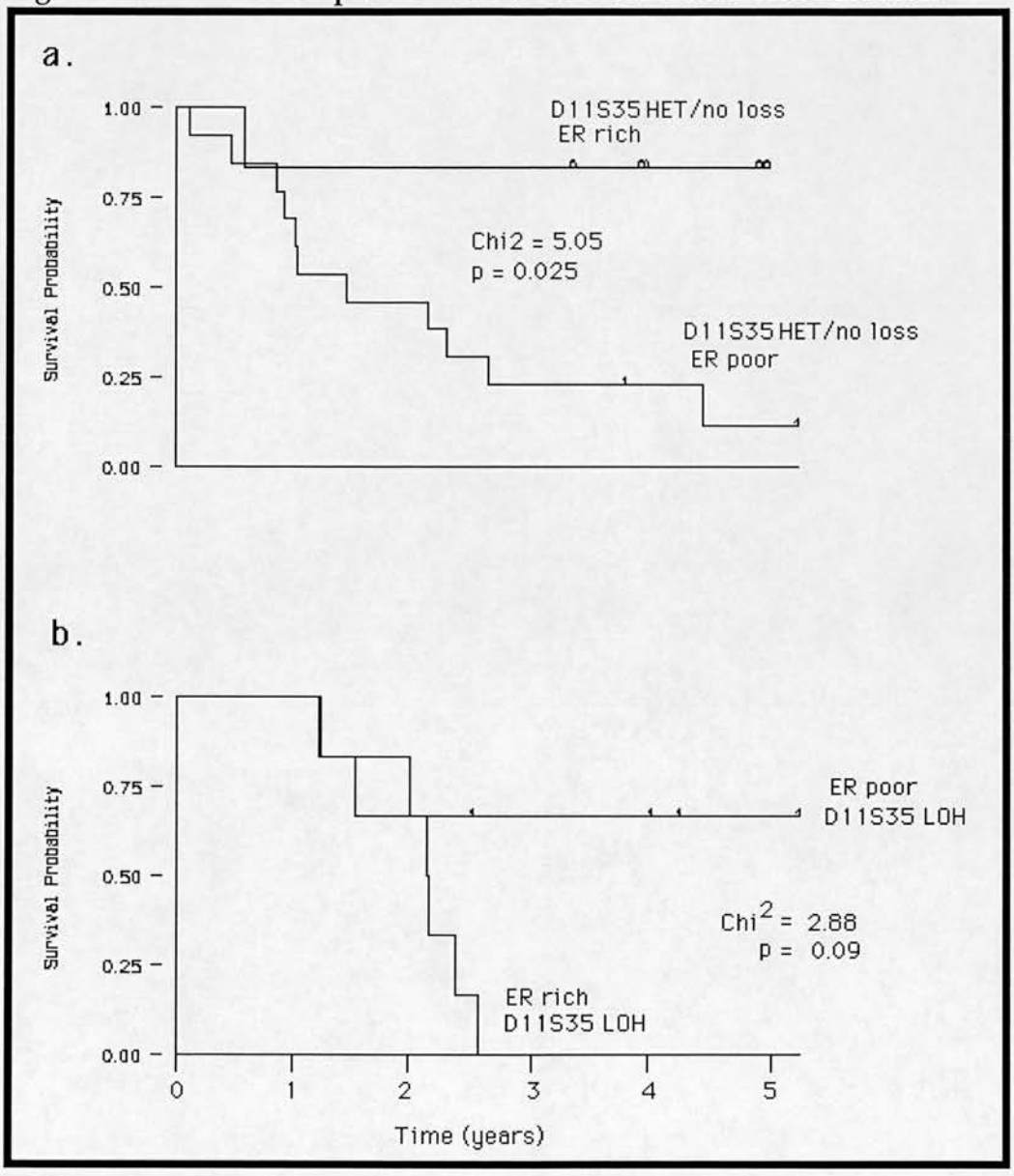



*Kaplan-Meier / log-rank analysis for (a) D11S35 heterozygous, no loss (HET/no loss) ovarian cancer patients subdivided by PgR content and (b) PgR-poor patients subdivided by D11S35 allele loss status.*

D11S35 heterozygous/no loss patients with ER-rich tumours (≥30 fmol/mg) had a better outcome compared to those with ER-poor (<30 fmol/mg) tumours (p=0.025) (Figure 3.15a). It is interesting that a paradoxical observation was noted those with LOH at D11S35: in this

group those who had ER rich tumours did particularly badly as compared with those who had ER poor tumours (Figure 3.15b).

**Figure 3.15 Relationship of D11S35 allele loss status and ER status**




Patients who had D11S35 constitutively heterozygous ER-rich (≥30 fmol/mg) tumours were then plotted according to whether they had LOH at D11S35 or not (see figure 6.1), and a significant (p=0.014) survival advantage in favour of those with no LOH at D11S35 was observed (although this subgroup comprised only 12 patients).

### 3.4 Detailed LOH analysis of 11q 23.3-q24.3 in ovarian cancer

#### 3.4.1 Rationale

In view of the 11q LOH findings described above, refinement of the LOH map of the 11q22-qter region in epithelial ovarian cancer was performed using selected tumours known to have loss somewhere on chromosome 11, concentrating on the sub-region of LOH identified above and associated with adverse survival(Figures 3.6 and 3.7).

Clinical and pathological characteristics of the patients selected for this part of the study are outlined in Table 3.13.

**Table 3.13 Clinicopathological characteristics of 11q study cohort**

| | | |
|---|---|---|
| **Number of patients** | | 40 |
| **Previously known to have Chromosome 11 LOH** | | 40 |
| **Ovarian adenocarcinoma (EOC)** | | 31 |
| **Histology** | Serous | 16 |
| | Endometrioid | 10 |
| | Mucinous | 4 |
| | Clear cell | 1 |
| **Differentiation** | Well | 2 |
| | Moderate | 12 |
| | Poor | 17 |
| **Stage** | I/II | 11 |
| | III/IV | 19 |
| | Not known | 1 |
| **Benign adenofibroma** | | 3 |
| **Borderline Malignant Potential** | | 3 |
| **Granulosa tumour** | | 2 |
| **Teratoma** | | 1 |

#### 3.4.2 Results of Molecular Analysis

LOH was detected somewhere on chromosome 11 in all 40 tumours in this series (selected for detailed analysis from our previous study). Several blood/tumour pairs (H59, G56, G43, H5, G47, H55, G42, G46 and G18) exhibited chromosome 11 LOH outside the 11q22-q25 region (see Figure 3.5).

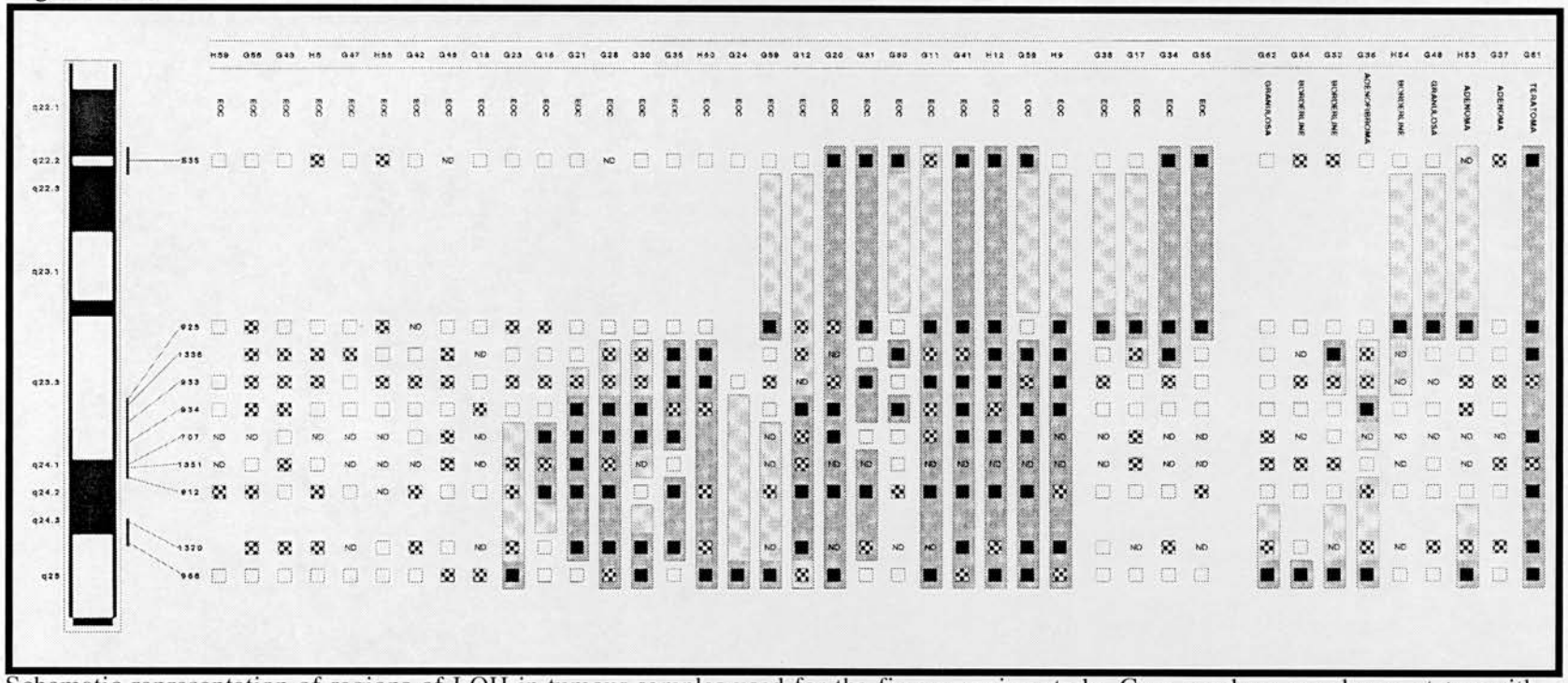
Figure 3.16 shows that two separate regions of LOH are identified within the 11q22-q25: the minimum extent of the telomeric region of

LOH (11q23.3-24.3) is defined by tumour G16. The centromeric extent of this region is defined by D11S934 and the telomeric extent is defined by D11S1320 (Figures 3.16 and 3.17). The size suggested for this region from a recently published radiation hybrid map is 169 centirays (about 8.5 Mb) (15). Within this region, the frequency of LOH at D11S912 was 11/19 (58%) EOCs and 0/5 benign/borderline; at D11S707 it was 9/13 (69%) in EOCs and 0/1 borderline. LOH involving this distal deletion unit occurred in 18/31 (58%) of EOCs.

The large centromeric region of LOH lies between D11S35 (11q22) and D11S925 (11q23.3) as defined by tumours G36, G17, G34 and G55 (Figure 1). LOH involving this centromeric deletion unit occurred in 13/31 (42%) of EOCs.

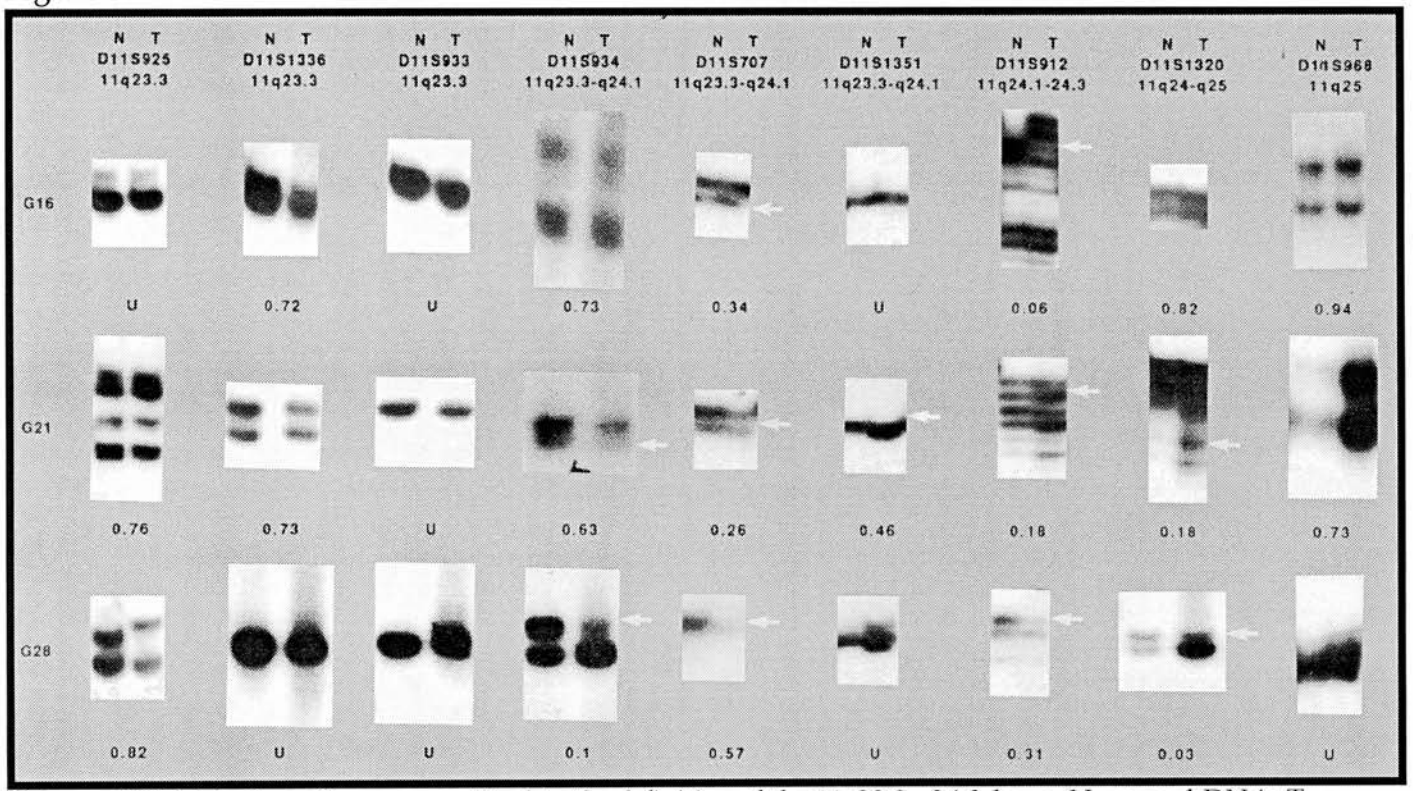
Only a minority of tumours exhibited LOH at either 11q22-q23.3 (4/31, 13%) or 11q23.3-24.3 (8/31, 26%) alone. Nearly 30% of the cases simultaneously exhibited LOH at both 11q22-q23.3 and 11q23.3-q24.3.

**Figure 3.16**




Schematic representation of regions of LOH in tumour samples used for the fime mapping study. Case numbers are shown at *top* with the histological diagnosis below. Microsatellite loci, used to detect LOH, are at the *left* of the diagram, and their approximate position is indicated on the chromosome 11 idiogram. *Shaded areas* correspond to regions of LOH. *Dark shadowing*, a region of secure LOH; *Light shadowing*, a region of uncertainty; *Unshadowed*, heterozygosity. *Light boxes*, loci maintaining heterozygosity without LOH; *checked boxes*, uninformative (homozygous) loci; *dark boxes*, LOH; *ND*, not determinable.

Figure 3.17




*Primary LOH data from three cases critical to the definition of the 11q23.3-q24.3 locus. N, normal DNA; T, tumor DNA shown at top from patients G16, G21 and G28 (left). Microsatellite loci are shown from centromeric (top left) to telomeric (top right). Arrows, alleles showing LOH (allele imbalance). Densitometric ratios of allele intensity were calculated (shown at bottom) and values between 0.0 and 0.7 are taken to indicate LOH. U, uninformative (homozygous). Note microsatellite instability at D11S925 for patient G28.*

### 3.4.3 Statistical Analysis of LOH in the telomeric 11q region

Fisher's exact test was used to determine if loss from these two regions was significantly associated with the clinicopathological features of ovarian cancer. No significant correlations were seen between regional losses and FIGO stage (p=0.15), histological type or differentiation grade. The centromeric 11q22-q23.3 region showed no statistical association with survival for regional LOH alone or in combination with other regions.

However LOH of the 11q23.3-q24.3 region either alone or in combination with LOH in other regions showed a strongly significant correlation with survival by Fisher's exact test (p=0.004) (alive versus dead patients with 33 month minimum follow-up), despite all the tumours in the series exhibiting LOH somewhere on chromosome 11, confirming our finding for D11S912 (see above).

Kaplan-Meier/ log-rank analysis showed no survival difference (p=0.632) for the centromeric region of LOH (Figure 3.18a) but the distal LOH region (11q23.3-q24.3) between D11S934 and D11S1320 correlated significantly with adverse actuarial survival (log rank test p=0.011) (Figure 3.18b).

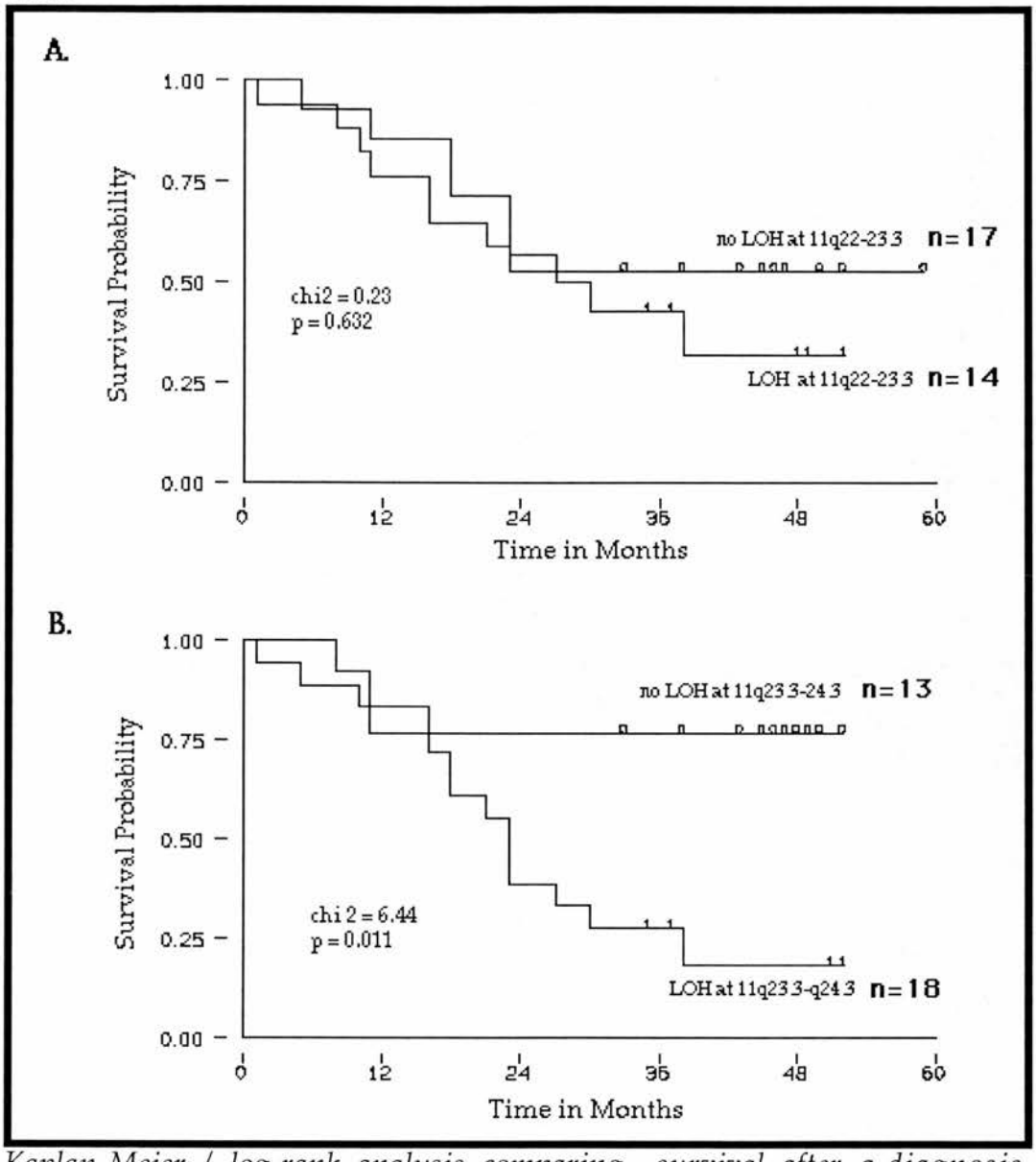
However, the log-rank analysis was not significant when only advanced FIGO stage cancers were considered in relation to 11q23.3-q24.3 LOH status (p=0.09).

### 3.4.4 Other tumour types

Data from a benign ovarian teratoma, regarded as haploid parthenogenetic demonstrating LOH at all informative chromosome 11 loci was included. This tumour also had clear LOH at all other informative chromosome 11 and 17 loci tested, and provides a useful positive LOH control.

Benign and borderline epithelial tumours showed no case with secure LOH involving the D11S934-D11S1320 distal SRO. The two granulosa cell tumours had minimal LOH located out-with the critical region.

**Figure 3.18 Survival of ovarian cancer patients according to LOH at each**




*Kaplan-Meier / log-rank analysis comparing survival after a diagnosis of ovarian cancer in (A) those with or without LOH at 11q22-q23.3 and in (B)those with or without LOH at 11q23.3-q24.3. LOH at 11q23.3-q24.3 is associated with significantly reduced survival (p=0.011, log-rank test) indicating an aggressive disease course.*

### 3.4.5 Further analysis of the distal 11q in ovarian cancer cell lines: A search for homozygous deletions

Having defined the distal 11q LOH locus, the possibility was considered that it might be homozygously deleted in ovarian cancer cell lines along the same lines as p16. In summary, no homozygous deletions were demonstrated for 6 polymorphic microsatellites from the telomeric 11q locus in 14 ovarian cancer cell lines.

**Table 3.14 Mapping the distal 11q LOH locus for evidence of homozygous deletions in ovarian cancer cell lines.**

| PRIMER-> SAMPLE | D11S934 | D11S707 | D11S1351 | D11S450 | D11S912 | D11S1320 |
|---|---|---|---|---|---|---|
| PEO1 | + | + | + | + | + | + |
| PEO6 | + | + | + | + | + | + |
| PEO14 | + | + | + | + | + | + |
| PEO16 | + | + | + | + | + | + |
| PEA2 | + | + | + | + | + | + |
| A2780 | + | + | + | + | + | + |
| OVCAR3 | + | + | + | + | + | + |
| OVCAR4 | + | + | + | + | + | + |
| OVCAR5 | + | + | + | + | + | + |
| 41M | + | + | + | + | + | + |
| 59M | + | + | + | + | + | + |
| OAW-28 | + | + | + | + | + | + |
| SKOV3 | + | + | + | + | + | + |
| OAW-42 | + | + | + | + | + | + |

+ = *allele(s) present by PCR*

# 4. MICROCELL MEDIATED CHROMOSOME TRANSFER

The preceding investigation showed that frequent LOH was present at specific regions on chromosome 11, suggesting the presence of tumour suppressor genes in these regions. Additionally, the lack of correlation of 11p and 11q LOH generally suggested that unlike chromosome 17, whole chromosome 11 loss is uncommon in EOC. This fragmentation with high rates of LOH at several discrete regions raises the possibility that several TSGs may be involved. The alternative explanation is that these regions do not contain TSGs and simply represent high frequency non-specific distal chromosomal disruption as a part of generalised genomic instability in ovarian cancer cells. This null hypothesis was somewhat tempered this analysis by the observation that LOH in individual tumours at the 11q sub-telomeric locus was restricted to an 8.5 Mb region with several examples of telomeric retention of chromosome.

Furthermore, these regions demonstrated co-loss with other polymorphic markers and were significantly associated with adverse clinicopathological features of the disease suggesting that they might house TSGs involved in ovarian tumour initiation and progression. These findings allowed a speculative multistep model of ovarian cancer development to be constructed (see discussion).

Clearly, a major reservation of such LOH analyses is that they do not address questions such as "Does disruption of a TSG in this region actually cause ovarian cancer ?" or "What component of progressive ovarian neoplasia is unmasked by disruption of this TSG ?" In order to address the issue of causation (in the absence of good candidates in these regions), and as part of an integrated programme to ultimately clone these genes, if they exist, I set out to transfer a single, normal human chromosome 11 into ovarian cancer cell lines. The objective was firstly to observe the effects that this would have, and secondly to develop resources from which I could attempt to clone such genes. This was a protracted and demanding process and a chapter on developing this method and its results is warranted.

As can be seen from the introduction, the ability to move specific chromosomes between cells is an extremely powerful tool which straddles cell function and gene position. However, MMCT (as I have discovered) is a demanding empirical technique; optimal conditions vary widely between different donor and different recipient cell lines, and re-optimisation is required for each donor and recipient cell line. Two rodent cell lines carrying a neo-tagged normal human chromosome 11 were used to achieve MMCT into ovarian cancer cell lines. Since this had not been previously achieved, optimisation of the method using 556.1.5, a mouse cell line bearing a human chromosome 11 as a reliable donor and HeLa, an established permissive chromosome recipient was performed initially.

### 4.1 Transfer of *hyg* and *neo* resistance to ovarian cancer lines

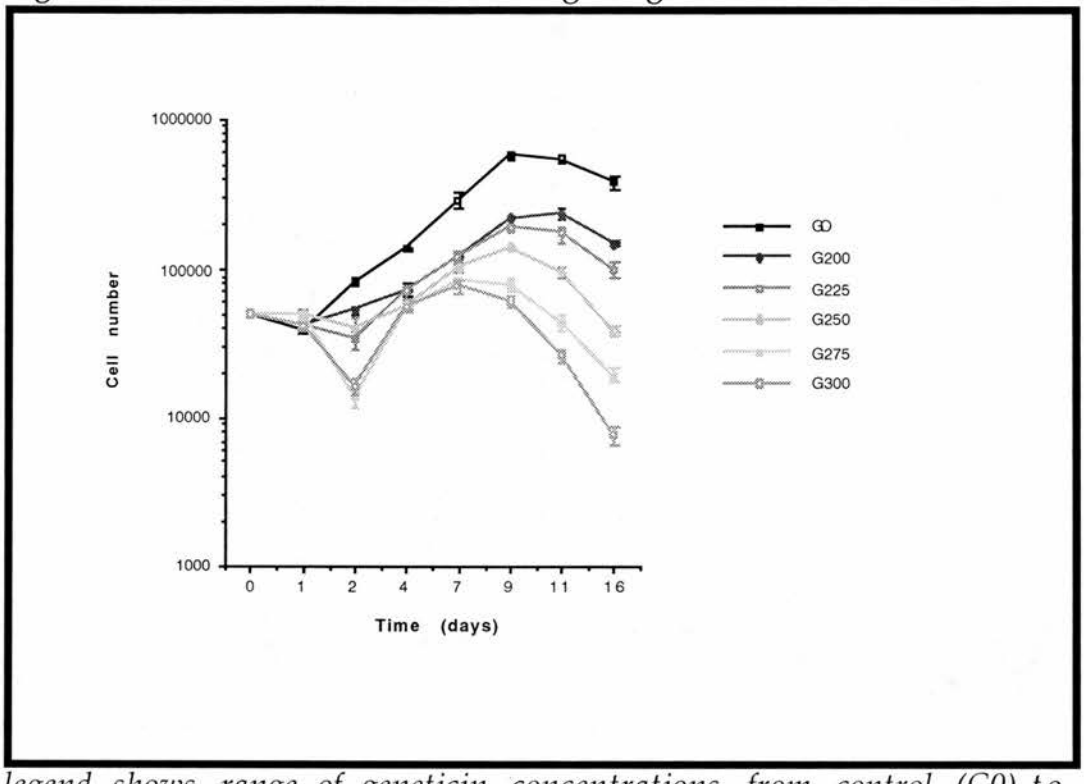
The first objective of this part of the work was to determine which ovarian cancer cell lines would be suitable for chromosome transfer. Since chromosome transfer had not been achieved in ovarian cancer at the time I commenced this work, there was no guiding empirical precedent. I reasoned (simplistically) that if a cell line would not take up a plasmid by transfection, it was unlikely to take up a whole chromosome. Additionally, it was important to derive *neo*-transfected control cell lines for subsequent functional assays (see later). Furthermore, it was also desirable to obtain a panel of ovarian cell lines with hygromycin resistance to provide a counter-selection to eliminate possible microcell donor contaminant cell lines during the microcell mediated chromosome transfer selection procedure.

Several ovarian cancer cell lines were characterised with respect to their toxicity profiles to geneticin (G418, G) and hygromycin B (hmB, H) in order to determine the most utilitarian lines. Figure 4.1 shows typical kill curves over a range of geneticin concentrations for the ovarian cancer cell line PEO1.

Plotting best-fit curves for cells at certain days allowed the definition of the optimal geneticin concentration to kill the parent cell lines over

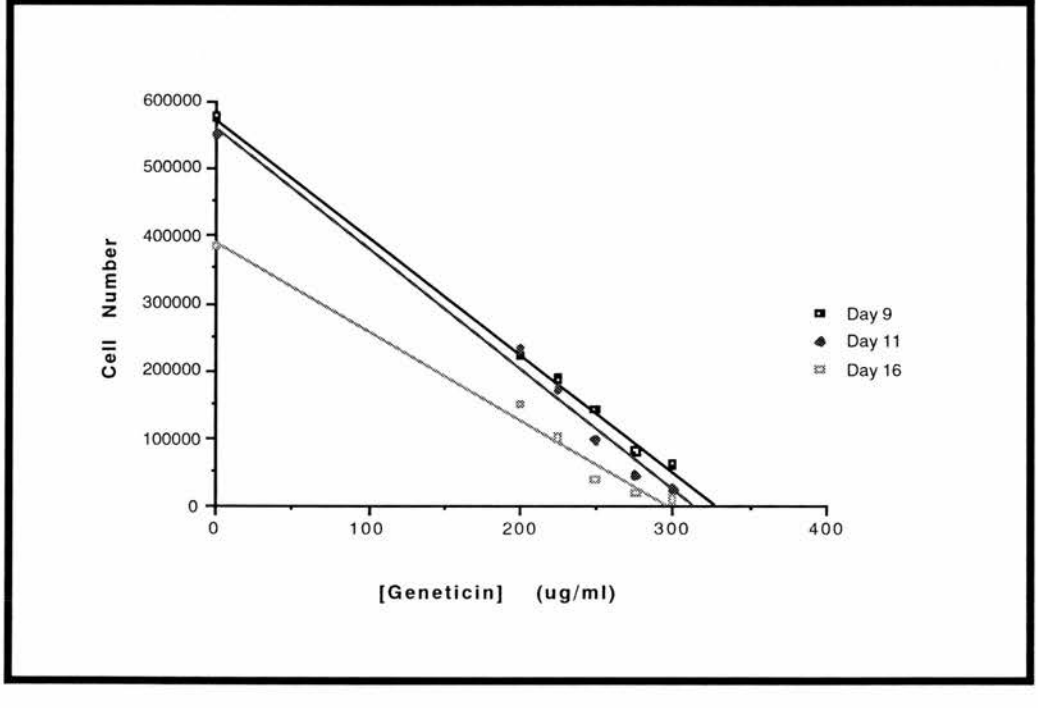
any defined time period, and these concentrations were typically very similar (Figure 4.2).

**Figure 4.1 PEO1 kill curves over a range of geneticin concentrations**




*legend shows range of geneticin concentrations from control (G0) to 300 μg/ml geneticin (G300). Y axis is a log-scale.*

**Figure 4.2 Simple curve fitting (best-fit linear regression) outlining optimal geneticin concentration for PEO1 cell kill**



Thus survival was empirically calculated for several ovarian cancer cell lines for both hmB and G418 marker (see Table 4.1). The time given is that to complete cell death of the cells at the specified concentrations of selection medium.

**Table 4.1 Optimal lethal concentrations of hmB and G418 for all lines used**

| Cell Line | Toxic [hmB] | Toxic [G418] |
|---|---|---|
| PEO1 | 75 μg /ml for 3 weeks | 300 μg /ml for 16 days |
| OVCAR3 | 50 μg /ml for 2 weeks | 325 μg /ml for 2 weeks |
| OVCAR4 | 100 μg /ml for 3 weeks | - |
| OVCAR5 | 100 μg /ml for 2 weeks | 400 μg /ml for 2 weeks |
| A2780 | 250 μg /ml for 10 weeks | 600 μg /ml for 2 weeks |
| HeLa (cervical) | - | 450 μg /ml for 2 weeks |

hmB and G418 resistance were induced in the ovarian cancer cell lines by lipofection of plasmids PUCY3/pTKHyg and pSVNeo respectively. All the microcell donors that we used transferred *neo*-resistance as the selectable marker for the chromosome. I attempted to confer hyg resistance on all ovarian lines in order to have counter selection against the rodent chromosome donor lines. Neo resistance was also transferred in order to provide $neo^r$ controls for subsequent functional assays. The lipofection conditions using the lipofection protocol (outlined in "methods", above) were established empirically for these cell lines and are outlined in Table 4.2

**Table 4.2 Optimal lipofection conditions for cell lines used**

| Cell Line | Lipofectin volume | Time |
|---|---|---|
| OVCAR3 | 10 μl | 6 hours |
| OVCAR5 | 20 μl | 12 hours |
| A2780 | 15μl | 12 hours |
| PEO1 | 20μl | 12 hours |
| HeLa | 15 μl | 6 hours |

Several cell lines were derived using this lipofection protocol, and these are summarised in Table 4.3

**Table 4.3 Clonally derived and pooled-clone cell lines obtained by plasmid transfection**

| Cell Line | $Hyg^r$ cell line | $Neo^r$ cell line |
|---|---|---|
| PEO1 | not transfectable | PEO-Neo (clonal) |
| OVCAR5 | not transfectable | not transfectable |
| OVCAR4 | not transfectable | not transfectable |
| A2780 | 278PH(pooled), 278PHX 278HC1, 278HC2 (both clonal) | 278N(clonal) |
| OVCAR3 | OH3 (clonal), OHX | ON3 (clonal) |
| HeLa | Not attempted | PHN(pooled), HN1,HN2, HN3 (all clonal) |

### 4.2 Optimisation of microcell production

Having established which cell lines were transfectable and therefore possibly capable of also taking up a whole chromosome, and having established the conditions for selection of the *neo* marker, the next component of the project was to optimise the production of microcells. Initially, J1 Cl4 B/B1.22 was used as a donor cell line. This is a CHO cell line with human chromosome 11 targeted with $neo^r$ at 11q23 using repeat-mediated homologous recombination (Watson et al, 1995).

The only information in the literature regarding quality control of microcells is that there is a "threshold" effect for the minimum number of microcells required for successful MMCT, but no subsequent increase in efficiency of generation of microcell hybrids with further increases in microcell numbers (Sanford and Stubblefield, 1987; Stubblefield and Pershouse, 1992). For this reason, no other surrogate endpoint to the protocol exists and one has to follow an

experiment for 6-8 weeks as a "self fulfilling prophecy" and record whether or not microcell hybrids are produced before one can comment on the quality of the microcells.

Two methods were attempted, developed by Stubblefield and co-workers (Sanford and Stubblefield, 1987; Stubblefield and Pershouse, 1992), and by Stanbridge and co-workers (Saxon and Stanbridge, 1987). The final protocol presented in the "methods" section ultimately represented a hybrid of the two.

### 4.2.1 The Stubblefield method

The first protocol that I attempted was developed by Stubblefield and co-workers (Stubblefield and Pershouse, 1992) and is attractive because it claims to be able to generate microcells from cells in mitotic arrest rather than requiring prolonged exposure to colcemid to promote the micronucleation as in the Stanbridge protocol. Additionally, in the Stubblefield protocol, microcells are actually formed in a percoll gradient in oak-ridge centrifuge tubes (rather than by placing the 25 $cm^2$ tissue culture flasks directly in the centrifuge as suggested in the Stanbridge protocol - this was felt to be somewhat hazardous).

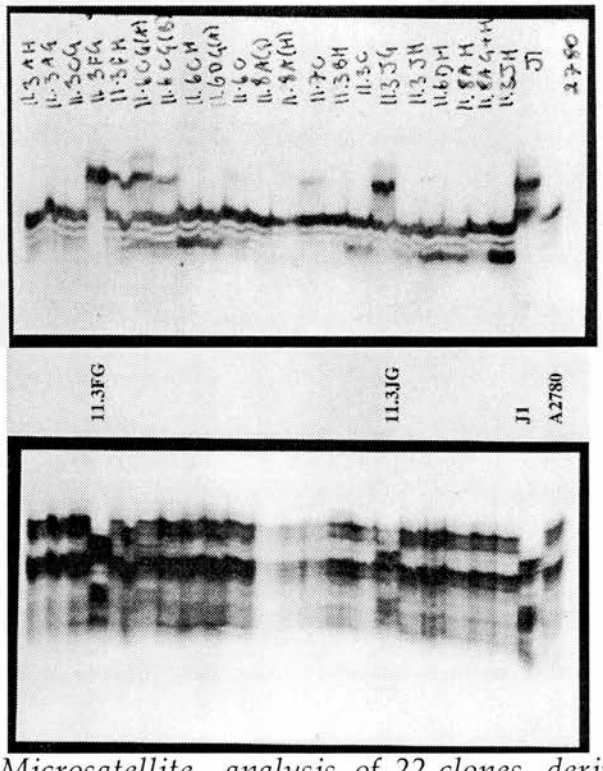
However despite these advantages and my persistence I report that this technique does not work in my hands after 28 experiments each observed for 6 weeks. These experiments are summarised below.

The initial experiment consisted of using the J1 donor (see above) to attempt to transfer chromosome 11 into A2780, a poorly differentiated ovarian cancer cell line (Eva et al, ). Microcells were formed using a protocol involving 4 hour incubation of colcemid (60 ng/ml), however no hybrids were obtained 6 weeks later. In order to try to achieve a threshold of viable microcells, methotrexate block was employed to increase the numbers of mitotically arrested cells in each experiment ($10^{-7}$M for 18 hours), but the next four experiments, all performed with the addition of methotrexate failed to produce hybrids. Methotrexate was then discontinued and colcemid incubations of

progressively 24 hours (2 experiments) and then 48 hours (2 experiments) also failed to yield hybrids at six weeks.

Methotrexate was then used with a 6 hour colcemid incubation, and a further two experiments generated a total of 77 clones, which was an order of magnitude more than the expected number of clones. 20 of these subsequently died in combined hmB/G418 dual selective medium (as outlined above). The remaining clones showed a mixture of morphologies of J1 and A2780. Clones picked from this experiment were positive by neo-PCR and polymorphic microsatellite markers were used to demonstrate chromosome transfer (Figure 4.3).

**Figure 4.3 Microcell fusion experiment 11**
**J1 donor microcells, A2780 recipient cells**




*Microsatellite analysis of 22 clones derived from MMCT experiment 11. Samples are identical for the top and bottom lanes. Top panel=D11S935 (11p13). Bottom panel= D11S912 (11q24.3). Far right lane= A2780 (ovarian cancer recipient cell line). Second from right= J1 Cl4 (CHO/human chr.11 somatic cell hybrid donor line).*

As can be seen from the above autoradiograph, two clones, 11.3FG and 11.3JG appeared to have taken up both alleles from the donor chromosome after microcell fusion.

Despite this apparent evidence of chromosome transfer these clones were ultimately shown to be contaminating co-cultures of donor and recipient cells which on *in-situ* hybridisation showed no evidence of chromosome transfer (data not shown, *in-situ* analysis performed by Dr. Vivienne Watson). This early lesson demonstrated that *in-situ* hybridisation was required for formal proof of chromosome transfer.

Following this, I adopted a "broad-spectrum" colcemid incubation time of 24 hours (based on Pershouse et al, 1993) and attempted to vary the temperature of the centrifuge to encourage the active cytochalasin-B mediated process of microcell extrusion, first at 34$^0$C and then at 37$^0$C. Neither temperature resulted in hybrid production. I then increased the time of exposure to polyethylene glycol (PEG) to fuse the microcells to the recipient line from 60s to 90s. Still, no hybrids were produced. These 28 failed experiments are summarised in Table 4.4, representing nearly 10 months of work.

**Table 4.4 Summary of "Stubblefield" protocol experiments**

| Experiment | Donor | Recipient | Method | Microcell yield | Selection | Result (6 weeks) |
|---|---|---|---|---|---|---|
| 1 | J1 (Chr.11) | A2780 parent | 4 hr. colcemid | | G600 | no hybrid clones |
| 2 | J1 (Chr.11) | A2780 parent | MTX, 4 hr. colcemid | | G600 | no hybrid clones |
| 3 | J1 (Chr.11) | A2780 parent | MTX, 6hr. colcemid | | G600 | clones grew after 6/52, senesced |
| 4 | J1 (Chr.11) | 278PH | MTX, 6hr. colcemid | | G600 | no hybrid clones |
| 5 | J1 (Chr.11) | 278PH | MTX, 6hr. colcemid | | G600 | no hybrid clones |
| 6 | J1 (Chr.11) | 278HC1 | 6 hr. colcemid | | G550/G450 | no hybrid clones |
| 7 | J1 (Chr.11) | 278HC1 | 24 hr. colcemid | | G550/G450 | no hybrid clones |
| 8 | J1 (Chr.11) | 278HC2 | 24 hr. colcemid | | G550/G450 | no hybrid clones |
| 9 | J1 (Chr.11) | 278PHX | 24 hr. colcemid | poor | G450 | no hybrid clones |
| 10 | A9:1 (Chr.1) | 278PHX | 24 hr. colcemid | | G450 | no hybrid clones |
| 11 | J1 (Chr.11) | 278PH | MTX, 6hr. colcemid | good | G450 | 57 clones, 2 distinct populations |
| 12 | A9:1 (Chr.1) | 278PH | MTX, 6hr. colcemid | good | G450 | 2 distinct clones: died |
| 13 | 278N | 278HC2 | 48 hr. colcemid | excellent | G450/G400 | no hybrid clones |
| 14 | A9:1 (Chr.1) | 278HC2 | 48 hr. colcemid | good | G450/G400 | no hybrid clones |
| 15 | J1 (Chr.11) | 278HC2 | 24 hr. colcemid/37C spin | good | G450/G400 | no hybrid clones |
| 16 | J1 (Chr.11) | 278PH | 24 hr. colcemid/37C spin | good | G450/G400 | no hybrid clones |
| 17 | J1 (Chr.11) | PEO1 | 24 hr. colcemid/37C spin | | G300 | no hybrid clones |
| 18 | J1 (Chr.11) | OVCAR3 | 24 hr. colcemid/37C spin | | G325 | no hybrid clones |
| 19 | J1 (Chr.11) | 278PH | 24 hr. colcemid/37C spin | | G450 | no hybrid clones |
| 20 | A9:1 (Chr.1) | PEO1 | 24 hr. colcemid/34C spin | | G300 | no hybrid clones |
| 21 | A9:1 (Chr.1) | OVCAR3 | 24 hr. colcemid/34C spin | | G325 | no hybrid clones |
| 22 | A9:1 (Chr.1) | 278PH | 24 hr. colcemid/34C spin | | G450 | no hybrid clones |
| 23 | J1 (Chr.11) | OVCAR3 | 48 hr. colcemid/34C spin | | G325 | no hybrid clones |
| 24 | 278N | 278PH5hr. | colcemid/34C spin | PEG 90s | G450 | no hybrid clones |
| 25 | 278N | 278PH5hr. | colcemid/34C spin | PEG 90s | G450 | no hybrid clones |
| 26 | J1 (Chr.11) | 278PH5hr. | colcemid/34C spin | PEG 90s | G450 | no hybrid clones |
| 27 | J1 (Chr.11) | 278PH5hr. | colcemid/34C spin | PEG 90s | G450 | no hybrid clones |
| 28 | J1 (Chr.11) | OH3 | 5hr. colcemid/34C spin | PEG 90s | G325 | no hybrid clones |

### 4.2.2 The Stanbridge method

Having failed to apply the Stubblefield method, an attempt was made to produce microcell hybrids using the Stanbridge method (Saxon and Stanbridge, 1987). With the difficulties encountered with the Stubblefield method, an alternative approach of using established microcell donor (556.1.5) and recipient (HeLa) lines were included to try and repeat Stanbridge's original experiment of chromosome 11 transfer to (and suppression of tumorigenicity of) the HeLa cell line. I performed these experiments as a visiting worker at the Molecular Carcinogenesis section of the Sylvius laboratory at the University of Leiden, the Netherlands as a guest of Dr Aswin Menke, Dr A.G. Jochemsen, and Prof. A. J. Van der Eb. In this protocol, the donor line is grown to 70% confluence and colcemid 50 ng/ml is added for 48 hours to induce micronucleation. Colcemid/media is replaced by warm serum free media with 20 μg/ml cytochalasin-B, and the bottles are filled to the neck. The Nunc flasks are placed in a Beckman rotor type 16 with 100 ml 37$^0$C water in each bucket and spun at 14,000 rpm/34$^0$C for 70 minutes. The colcemid/media is removed and the microcell pellets are resuspended in 12 ml serum free media. This suspension is then sequentially filtered through 8, 5 and 3 μm Nuclepore polycarbonate filters, spun down and the microcell pellet is resuspended in 2.5 ml serum free medium (HEPES buffered) with 50 μg/ml PHA-P. 2.0 ml of this suspension is layered on to the receptor cells and the remaining 0.5 ml is plated in a dish to observe for contamination by viable donor cells. The microcells attach for 20 minutes at room temperature. The medium is then carefully removed and 2 ml warm PEG is added. Fusion is allowed to occur for exactly 60 seconds and then the cells are washed three times in serum free medium. Fresh 10%FCS media is added and the cells are grown without selection for 3 days to recover. The cells are split 1:8-1:10 and after a further 24 hours, selection is applied. Twenty-one experiments were performed with this technique and these are summarised in

Table 4.5. Unfortunately the vast majority of experiments developed yeast infections. One experiment, however, did produce viable clones from which had the appearance of HeLa which were neo resistant. However, these also came down with late fungal infection, but it was clear that I had managed to optimise conditions using previously reported recipient 556.1.5 and HeLa (Saxon et al, 1986)

**Table 4.5 Summary of "Stanbridge" protocol experiments**

| Experiment | Donor | Recipient | Method | Result (6 weeks) |
|---|---|---|---|---|
| 29 | J1 (Chr.11) | OH3 | 3 um filter | yeast infection |
| 30 | 556.1.5 (Chr.11) | OH3 | 3 um filter | yeast infection |
| 31 | J1 (Chr.11) | OH3 | 3 um filter | yeast infection |
| 32 | J1 (Chr.11) | OH3 | 3 um filter | yeast infection |
| 33 | J1 (Chr.11) | 278PH | 3 um filter | cell death ++ |
| 34 | J1 (Chr.11) | 278PH | 3 um filter | yeast infection |
| 35 | J1 (Chr.11) | 278PH | 5 um filter | yeast infection |
| 36 | J1 (Chr.11) | 278PH | 3 um filter | yeast infection |
| 37 | J1 (Chr.11) | 278PH | 3 um filter | yeast infection |
| 38 | J1 (Chr.11) | 278PH | 5 um filter | yeast infection |
| 39 | 556.1.5 (Chr.11) | 278PH | 3 um filter | cell death ++ |
| 40 | 556.1.5 (Chr.11) | 278PH | 3 um filter | yeast infection |
| 41 | 556.1.5 (Chr.11) | 278PH | 3 um filter | yeast infection |
| 42 | 556.1.5 (Chr.11) | 278PH | 3 um filter | no clones |
| 43 | 556.1.5 (Chr.11) | 278PH | 3 um filter | no clones |
| 44 | 556.1.5 (Chr.11) | 278PH | 5 um filter | no clones |
| 45 | 556.1.5 (Chr.11) | 278PH | 3 um filter | no clones |
| 46 | 556.1.5 (Chr.11) | 278PH | 5 um filter | no clones |
| 47 | 556.1.5 (Chr.11) | HeLa | 5 um filter | CLONES PRODUCED |
| 48 | 556.1.5 (Chr.11) | 278PH | 5 um filter | no clones |
| 49 | J1 (Chr.11) | 278PH | 5 um filter | no clones |

## 4.3 Development of high volume throughput MMCT.

### 4.3.1 Definition of a robust repeatable MMCT protocol

Experiment 47 (Table 4.5) established the optimal conditions for transfer of chromosome 11 from 556.1.5 into HeLa (using a 5μm filter). Back in Edinburgh, in order to achieve optimal monochromosome transfer I repeated the experiment using a 3μm filter which further limits the maximum size of the microcells. However, for convenience I coupled the micronucleation and fusion conditions of the Stanbridge protocol to the higher throughput component of the Stubblefield protocol for generating microcells. Stubblefield's percoll/cytochalasin-B method in oak ridge tubes allows one to generate up to 4 different

microcell preparations per day, whereas the Stanbridge method limits the worker to performing only 1-2 microcell preparations per working day.

From this first modified protocol two *neo*-resistant microcell hybrid clones appeared 3 weeks after G418 selection.

### 4.3.2 Definition of utilisable microcell donor and recipient cell lines

Having failed to generate hybrids between the J1 chr.11 donor line and ovarian cancer lines using the standard Stubblefield or Stanbridge protocols, I directly compared the ability of chr.11-containing microcells derived from either J1 or 556.1.5 to successfully produce microcell hybrids in HeLa under carefully controlled identical conditions. Under identical micronucleation conditions (75 ng/ml colcemid for 48 hours), the J1 donor produced far fewer micronuclei than 556.1.5. However both lines produced good yields of microcells after 3μm filtration, although the micronuclei produced by 556.1.5 were larger. The microcell preparations were simultaneously fused into two identical sister HeLa recipient plates seeded 24 hours previously which had reached 60% confluence immediately prior to fusion. Fusion was performed under identical conditions using the same solutions. Under these identical conditions, microcells generated from 556.1.5 produced microcell hybrid clones whereas those generated from J1 Cl4 did not (see Table 4.6)

**Table 4.6 Comparison of 556.1.5 and J1 Cl4 as chromosome 11 donor cell lines using a high-throughput optimised MMCT protocol**

| | 556.1.5 | J1Cl.4 B/B 1.22 |
|---|---|---|
| Colcemid | 75 ng/ml | 75 ng/ml |
| Spin speed/temp | 18.25K/32C | 18.25K/32C |
| Microcell yield | good(large) | good(smaller) |
| Recipient/split | HeLa/1:8 (60%) | HeLa/1:8 (60%) |
| PEG fusion time | 1 min. | 1 min. |
| recovery time | 3 days | 3 days |
| Selection | G450 | G450 |
| Hybrids/when? | 2 clones (3 weeks) | no clones (8 weeks) |

Having established that J1 was not a suitable donor, an extensive attempt was made to transfer chromosome 11 from 556.1.5 into the

available cell lines that I had. In summary, of the cell lines that I used as recipients, only OVCAR3 and its derivative clonal lines served as suitable recipients for transfer of chromosome 11 (see Table 4.7)

**Table 4.7 Optimised MMCT protocol applied for chromosome 11 donor cell lines and ovarian cancer cell lines**

| Experiment | Donor | Recipient | Result by 8 weeks |
|---|---|---|---|
| 50 to 55 | 556.1.5 | HeLa | 52 clones from 3 experiments |
| 56 and 57 | J1Cl.4 | OH3 | no clones |
| 58 to 61 | 556.1.5 | OH3 | 6 clones from 2 experiments |
| 62 to 65 | 556.1.5 | PEO1 | no clones |
| 66 and 67 | 556.1.5 | A2780 | no clones |
| 68 | 556.1.5 | PEO14 | no clones |
| 69 to 72 | 556.1.5 | OHX | 9 clones from 3 experiments |

*OHX is a derivative line of OH3 which was passaged once as a xenograft in a SCID mouse and rescued back to improve take rates of OH3*

The microcell hybrid clones generated from the above experiments were picked, expanded, and stored in liquid nitrogen. They were then mapped extensively for evidence of chromosome 11 transfer (see below) prior to functional analysis for evidence of tumour suppressor effects (see chapter 5). During expansion of the first HeLa microcell clone, the line underwent growth arrest and the original cells died. However, surviving revertants were observed and 38 of these were picked and expanded for further characterisation in the hope that reversion was associated with discrete disruption of a region of the donated chromosome 11 locus. Table 4.8 shows a list of chromosome 11 microcell hybrid cell lines generated. As a general guide, the naming of the microcell hybrid clones consists of prefixing with the chromosome donated, then an abbreviation of the recipient line, and finally a suffix consisting of the experiment and clone number.

**Table 4.8 Chromosome 11 microcell hybrids generated in this study**

| Clones | Donor | Recipient | Comments |
|---|---|---|---|
| 11H1 r1-20 | 556.1.5 | HeLa | surviving revertant lines |
| 11H2.1-10 | 556.1.5 | HeLa | |
| 11H3.1-10 | 556.1.5 | HeLa | |
| 11OH1 | 556.1.5 | OH3 | |
| 11OH2.1-4 | 556.1.5 | OH3 | |
| 11OHX1.1-3 | 556.1.5 | OHX | more tumorigenic than OH3 |
| 11OHX2.1-3 | 556.1.5 | OHX | |
| 11OHX3.1-3 | 556.1.5 | OHX | |

### 4.4 Generation of appropriate controls

Having obtained microcell hybrids containing chromosome 11 it was necessary to demonstrate that any observed functional effects were not simply due to clonal selection (even though the ovarian line OH3 was clonal by virtue of hygromycin transfection prior to microcell fusion). Two avenues were employed to obtain appropriate controls. The first was transfection of pSVneo to HeLa, OH3 and OHX.

In the case of HeLa, lines consisting of both pooled transfection (PHN, see Table 4.9) and clonal lines (HN1-3) were generated and compared with the parent cell line to ensure the lines behaved representatively. Transfer of human chromosomes 1, 11 and 12 from rodent donors to human cervical and ovarian cancer recipients was performed

In the case of OH3 and OHX, a series of clonal *neo*-resistant sub-lines were generated (OHN1-3 and OHXN1-3) and these did behave like the parent lines in functional assays and growth patterns (see chapter 5).

**Table 4.9 *neo*-transfected control cell lines**

| Recipient Line | *neo* transfected lines | behaviour |
|---|---|---|
| HeLa | PHN, HN1-3 | as parent |
| OH3 | OHN1-3 | as parent |
| OHX | OHXN1-3 | as parent |

Although these addressed the issue of clonal selection, they did not address the possibility that non-specific chromatin transfer effects could suppress growth, and it was therefore necessary to generate microcell hybrid clones that behaved identically to the parent but contained a "neutral" chromosome. Since many chromosomes are implicated by LOH and cytogenetic studies as housing potential TSGs, the choice of which chromosome to use in order to generate a control microcell hybrid line containing a transferred "neutral" chromosome was based simply on reviewing the LOH/cytogenetic evidence and identifying chromosomes infrequently involved, determining which of these were available as monochromosome somatic cell hybrids which had been used as donors and then obtaining these for microcell fusion. Chromosome 1 ( A9(neo-1)-4 from Dr Carl Barrett) and chromosome 12 ( 12-neo from Drs Carlo Conti and Carl Barrett) donors were obtained and MMCT was attempted with these donor lines. The results of these fusions is outlined in Table 4.10 below.

**Table 4.10 Chromosome 1 and 12 MMCT**

| Experiment | Donor | Recipient | Result by 8 weeks |
|---|---|---|---|
| 73-76 | A9 (neo-1)-4 | HeLa | 1 clone senesced at 8 weeks |
| 77-84 | A9 (neo-1)-4 | OH3 | 2 clones senesced at 7 weeks |
| 85-88 | 12-neo | OH3 | 3 clones from 3 experiments |
| 89-92 | 12-neo | OHX | 4 clones, all senesced |

As can be seen from the above table, chromosome 1 and 12 both exhibited senescence effects. Furthermore analysis of the remaining viable chr. 12/OH3 hybrids showed that there were functional growth suppression effects associated with introduction of chromosome 12 (see chapter 5 for description of data) and it was therefore unsuitable as a control. In view of this difficulty, an alternative approach was adopted. Reasoning that a good source of a chromosome without a functionally active tumour suppressor gene for the OH3 cell system

would be the parent cell line from which it was derived, a *neo*-resistant OVCAR3 sub-line was generated by transfection (ON3, see Table 4.3 above) and this was used as a microcell donor to transfer an unidentified (but by definition non-suppressing) chromosome by MMCT from ON3 to OH3. This was successfully achieved for both OH3 and OHX (see Table 4.11 and molecular analysis below) These cell lines behaved as the parents from which they were derived.

**Table 4.11 generation of control microcell hybrid clones**

| Experiment | Donor | Recipient | Result by 3 weeks | Behaviour |
|---|---|---|---|---|
| 93 | ONH | OH3 | 7 clones, 3 picked | ONOH1-3, as parent |
| 94 | ONH | OHX | 10 clones, 3 picked | ONOHX1-3, as parent |

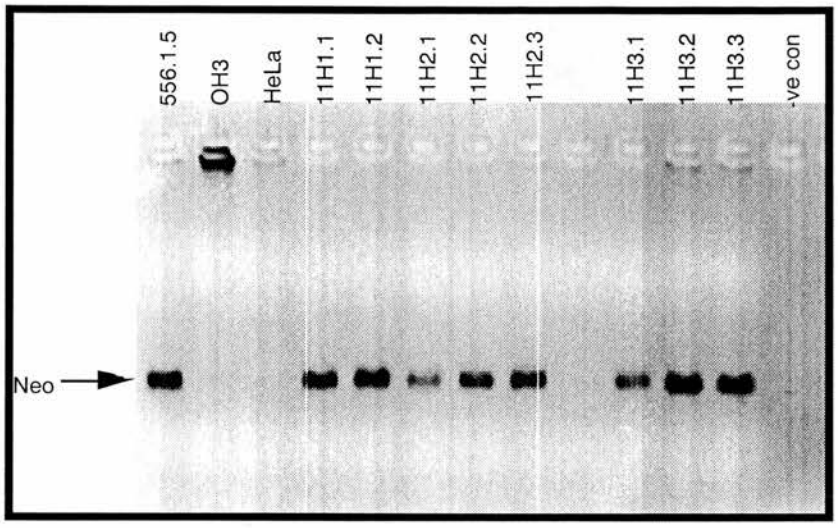
## 4.5 Molecular and cytogenetic analysis of microcell hybrids

### 4.5.1 Transfer of chromosome 11 to HeLa

#### *4.5.1.1 Confirmation of transfer of selectable markers*

As mentioned previously, HeLa was used as a well recognised recipient to develop the MMCT method and act as proof of principle that MMCT could be performed. Extensive analysis with HeLa was not performed with HeLa hybrids although many clones were generated and several unexpected conclusions were arrived at. After fusion of chromosome 11 microcells to a 70% confluent monolayer of HeLa, several clones grew in the fusion plates at a time when all HeLa cells had died after exposure to G418 in control plates. At this time, clones were picked and expanded, and DNA was prepared from them whilst still in 24 well plates. *Neo* PCR was initially performed to confirm that transfer had occurred. Although providing good circumstantial evidence, this confirmation is not however formal since it is possible for clones of apparently recipient morphology to grow in supportive co-culture with background contamination of donor cell lines(see Figure 4.3, above). Formal evidence of microcell transfer should therefore rest on *in-situ* analysis (see below). Figure 4.4 shows detection of transfer of *neo* sequence from 556.1.5 to HeLa in a series of clones derived from 3 separate microcell experiments.

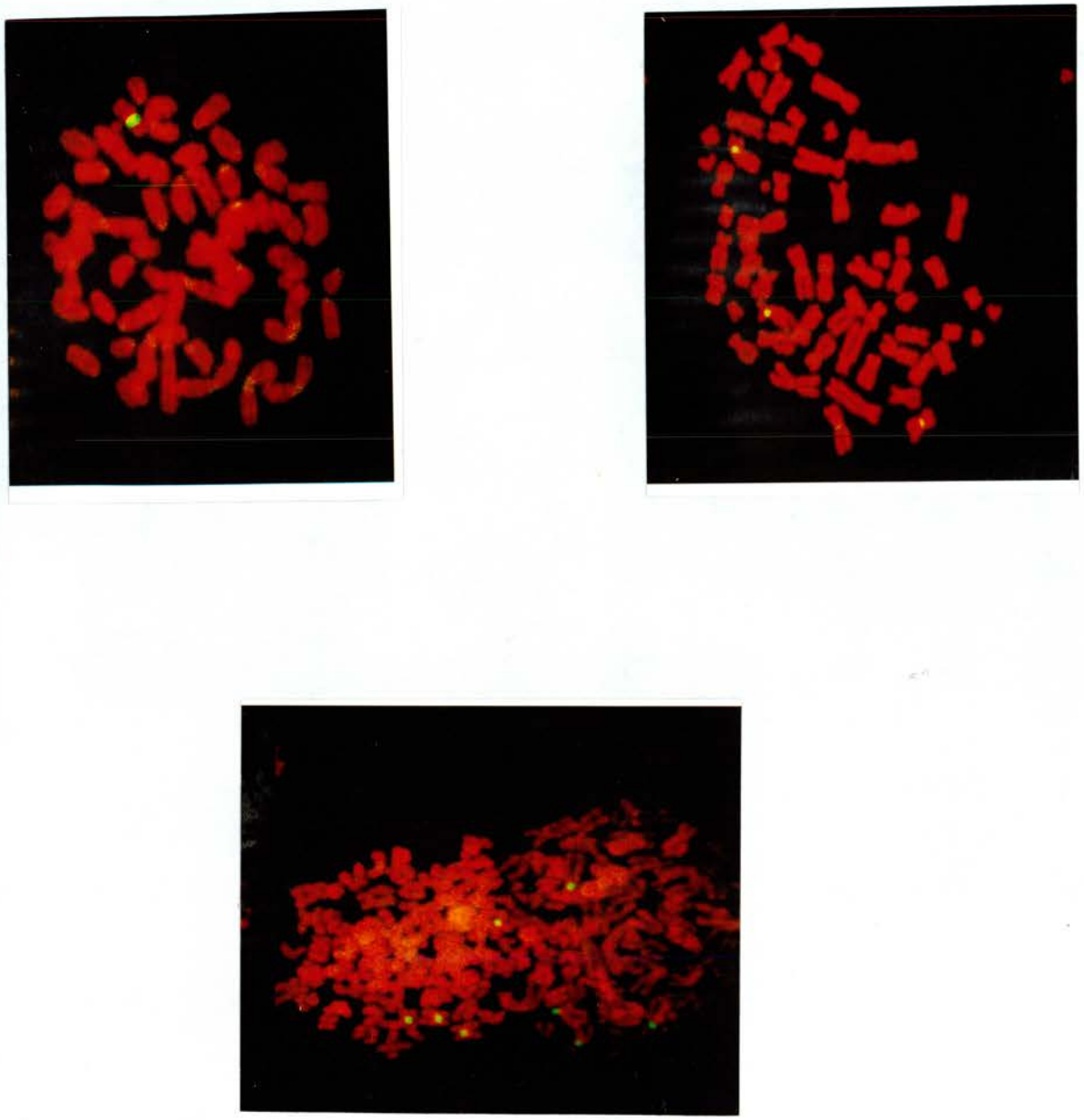
**Figure 4.4 detection of *neo* sequence by PCR in chromosome 11/Hela microcell hybrids.**




### *4.5.1.2 In-situ hybridisation*

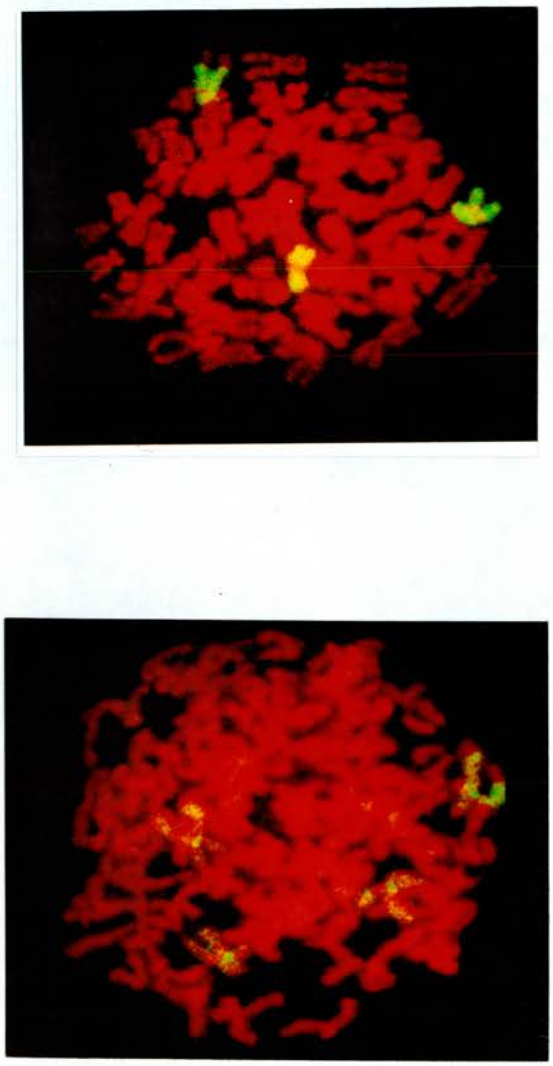
As mentioned above, *in-situ* analysis provides formal proof of MMCT and figure 4.5 shows Primed *in-situ* hybridisation (PRINS) using chromosome 11 centromeric alphoid primers. 556.1.5, HeLa and the microcell hybrid clone ... are shown in this example, with an increase in chromosome 11 centromeric signals in the microcell hybrid relative to HeLa. Figure 4.6 shows chromosome 11 *in-situ* painting with an extra whole chromosome 11 within the hybrid. HeLa/chromosome 11 microcell hybrids generally appear to take up the whole chromosome both by cytogenetic/*in-situ* analysis and also by microsatellite analysis.

**Figure 4.5 Primed *in-situ* hybridisation of HeLa and HeLa/chr 11 microcell hybrid 11H2.3**



*human chromosome 11 specific centromeric alphoid primers demonstrate one more chromosome 11 centromere in the microcell hybrid (bottom) than in HeLa (top right): this represents formal proof of chromosome transfer to HeLa from 556.1.5 (top left)*

**Figure 4.6 Chromosome 11 specific painting of HeLa and HeLa/chr 11 microcell hybrid 11H2.3**

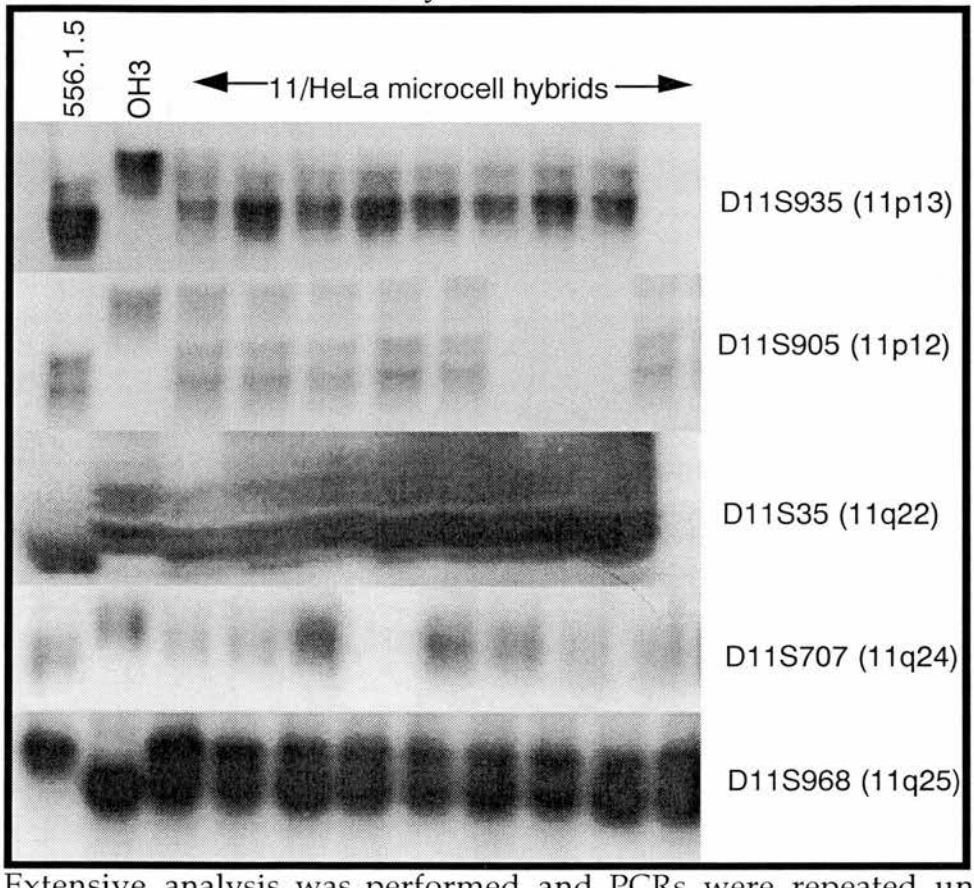


*Chromosome painting shows three copies of chromosome 11 in HeLa (top panel). Microcell hybrid 11H2.3 shows four copies of chromosome 11 confirming microcell mediated chromosome transfer*

*4.5.1.3 Polymorphic microsatellite analysis*

Using the same microsatellites used in the LOH analysis of the preceding chapter, DNA from the 556.1.5 donor, HeLa recipient and microcell hybrids was examined to see which regions of the transferred chromosome were retained or lost. Figure 4.7 shows examples of transferred alleles in the chr 11/HeLa hybrids.

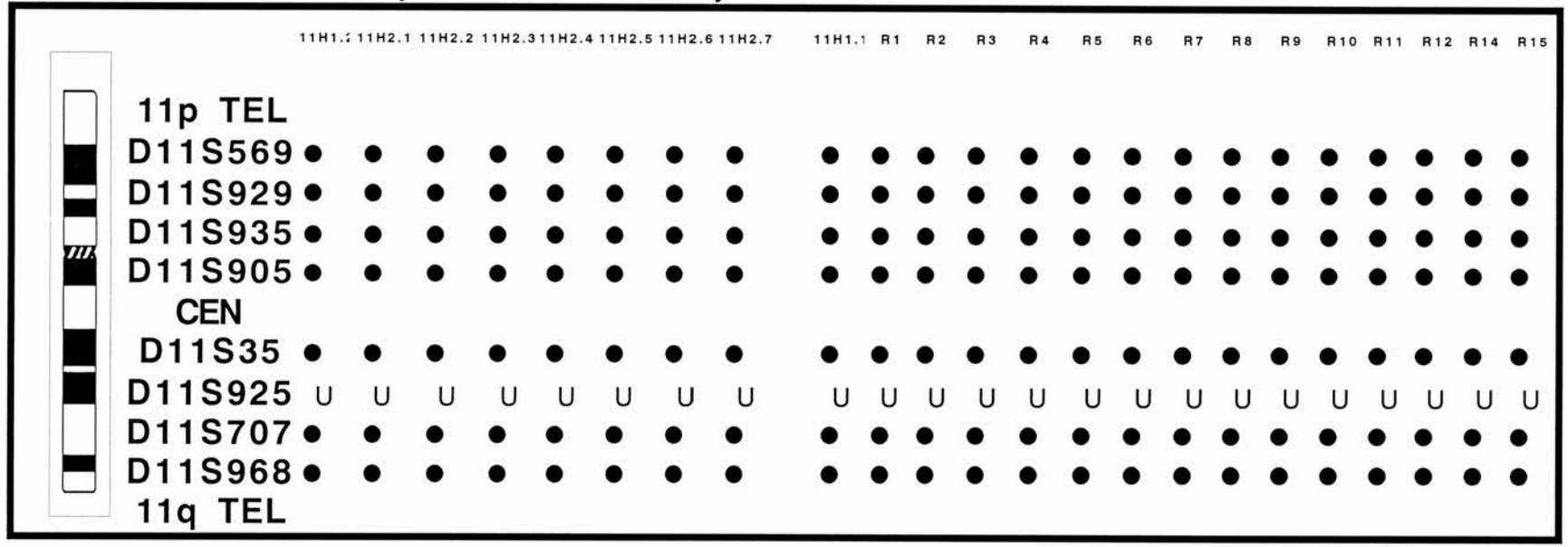
**Figure 4.7 Examples of microsatellite analysis in donor, HeLa recipient and chr 11/HeLa microcell hybrids**




Extensive analysis was performed and PCRs were repeated until all results were confirmed. Figure 4.8 presents an overview of analysis of all clones tested.

In summary, no deletions of the transferred chromosome were detected in HeLa microcell hybrids and this situation was in marked contrast to the results obtained with OVCAR3 (see below). Several viable, rapidly growing revertants picked from senescent clone 11H1.1 (see chapter 5) were analysed and found to contain all informative markers tested.

Figure 4.8 Microsatellite analysis of HeLa microcell hybrid clones



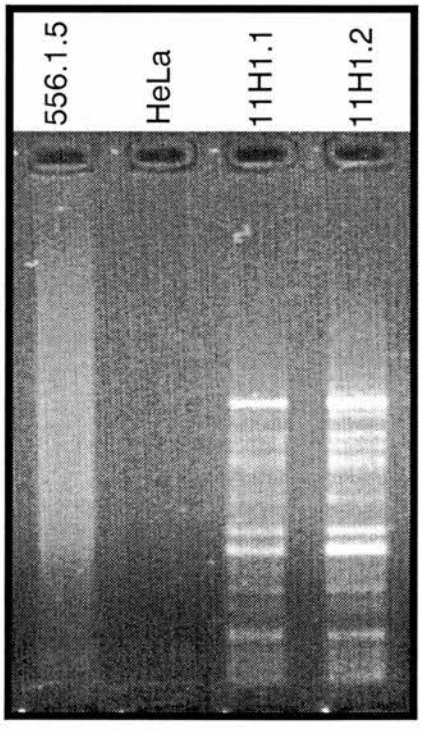

Schematic representation of analysis of transferred chromosome 11 alleles from 556.1.5 to HeLa recipient. Microcell hybrids are shown at *top*. Hybrid 11H1.1 and revertants isolated from this line are shown on the right of the diagram. Microsatellite loci used are shown at *left*. Their approximate position is indicated with respect to the chromosome 11 idiogram.

•, donor allele transferred to recipient hybrid clone. U, donor and recipient alleles identical, therefore uninformative at that locus.

#### *4.5.1.4 Interspersed repetitive sequence (IRS) PCR: co-transfer of murine sequences.*

Reports of microcell fusion have universally commented on the lack of mouse chromosome co-transfer in MMCT experiments using cytogenetic analysis. I reasoned that small fragments of mouse chromosomes could be transferred and not detected since they would be beyond the limits of resolution of classical cytogenetics, especially in a bizarre cytogenetic background of a malignant cell. PCR primers for L1 and B1 mouse repetitive sequences were used together to produce a murine fingerprint. PCR analysis indeed showed that co-transfer of cytogenetically tiny murine fragments was ubiquitous (figure 4.9), and this was of importance since these fragments could potentially interfere in a difference analysis cloning strategy (one of the ultimate aims of generating these hybrids).

**Figure 4.9 L1/B1 IRS-PCR of HeLa hybrids**



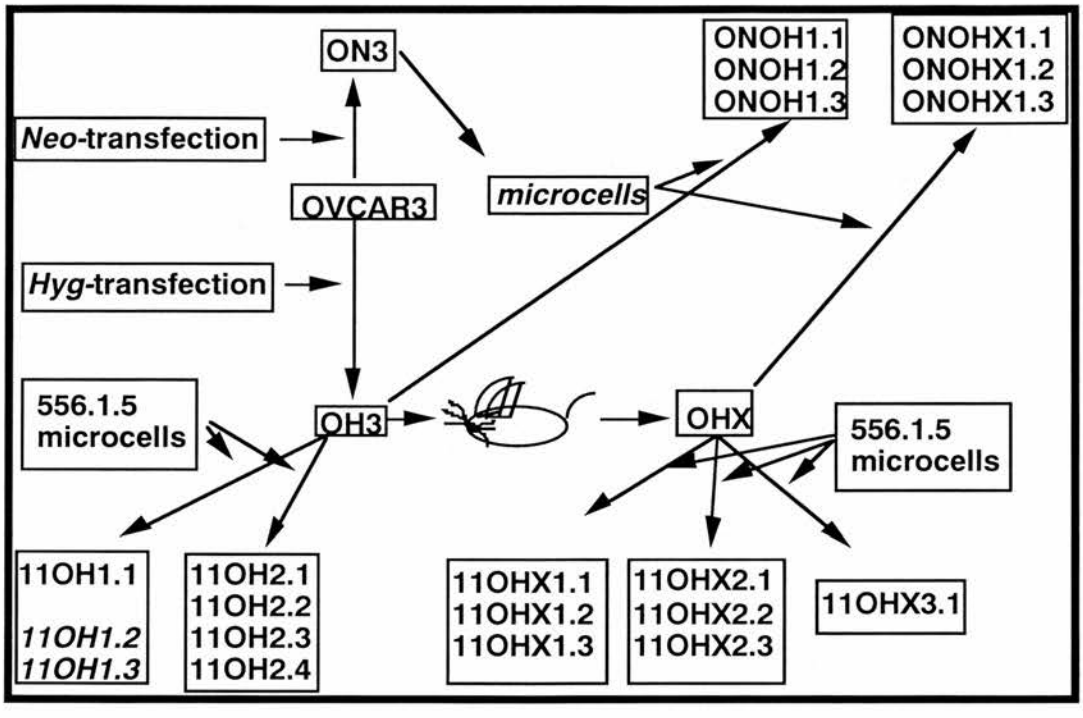
As can be seen from figure 4.9 above, two clones derived from the same microcell fusion experiment exhibited identical murine IRS-PCR fingerprints. This method of fingerprinting was extremely useful in determining whether clones from the same microcell experiment represented separate fusion events (until now an assumption made in most microcell fusion reports). With only few exceptions, all clones generated within a single experiment were identical with respect to their murine fingerprints suggesting a single initial MMCT event. This observation was utilised to great effect in cases where clones with differing behaviour, identical IRS-PCR patterns and differently fragmented donated human chromosomes were identified, and these represent valuable gene cloning resources (see below).

Evidence from mouse cot-1 *in-situ* painting (see next section) suggested that certainly in early passages, mouse fragments were seen as small autonomously replicating structures and were not integrated either into the donor human chromosome 11 or rearranged into the recipient genome. Mouse cot-1 painting was not performed for later passages of microcell hybrid lines, so no comment can be made about the subsequent behaviour of these co-transferred murine fragments.

### 4.5.2 Transfer of chromosome 11 to OVCAR3

The genealogy of microcell hybrids derived from the OVCAR3 recipient is shown in figure 4.10.

**Figure 4.10 Genealogy of OVCAR3 microcell hybrid lines generated by fusion with chromosome 11 and "neutral" control chromosome microcells.**



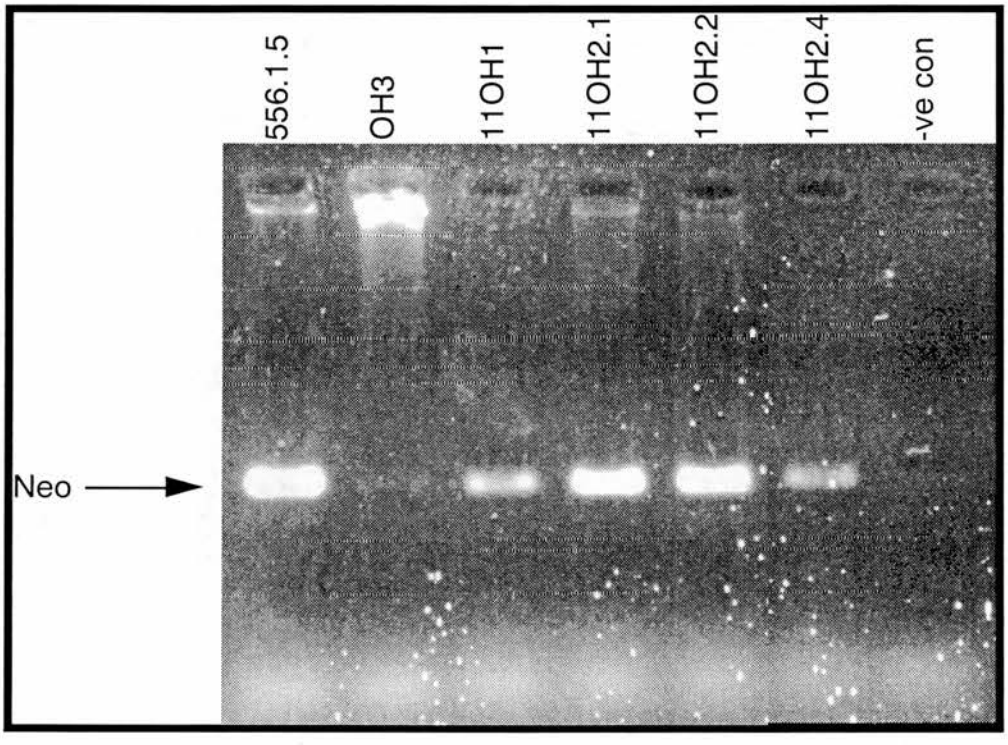

*Microcell clones 11OH1.2 and 11OH1.3 were derived as single cell clonal cell lines from (and within 3 passages of derivation of) the cell line 11OH1.1 from its original clone. ONOH and ONOHX represent ideal "neutral chromosome" MMCT controls*

#### *4.5.2.1 Confirmation of transfer of selectable markers*

Clones with clear OVCAR3 morphology were derived from several microcell experiments as shown above and DNA prepared from these clones was initially used for *neo*-PCR, providing good evidence of microcell mediated chromosome transfer.

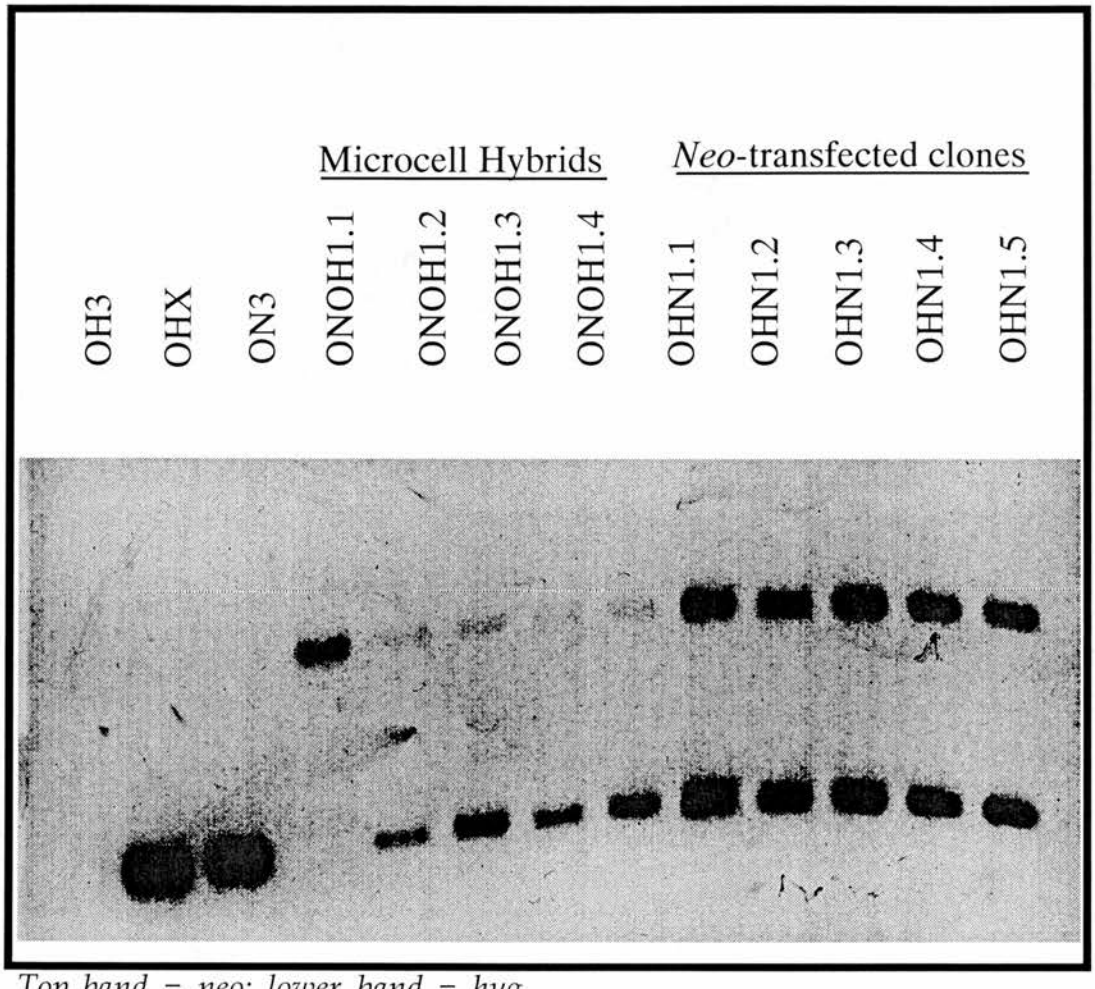
Figure 4.11 shows examples of *neo*-PCR for the 11OH series of microcell hybrids generated by MMCT from 556.15 to OH3 (*hyg*-transfected OVCAR3).

**Figure 4.11 *Neo*-PCR of OH3 derived chromosome 11 microcell hybrid clones**




Similarly figure 4.12 shows *neo* and *hyg* PCR of clones derived from control microcell experiments transferring a "neutral" *neo*-tagged chromosome from ON1 to OH3 ( ONOH); and *neo*-plasmid transfected OH3 (OHN). As can be seen, weak PCR bands representing single copy detection of *neo,* derived from microcell mediated chromosome transfer can be seen in comparison with multi-copy *neo* and *hyg*-detection observed in DNA derived from plasmid transfected cells. These control cell lines were important in the functional analysis subsequently performed since they behaved as the parent cell line despite having taken up *neo*-plasmid or a tagged "neutral" chromosome transferred from by MMCT.

**Figure 4.12 Control OH3 clones derived by neutral chromosome transfer and *neo*-transfection**

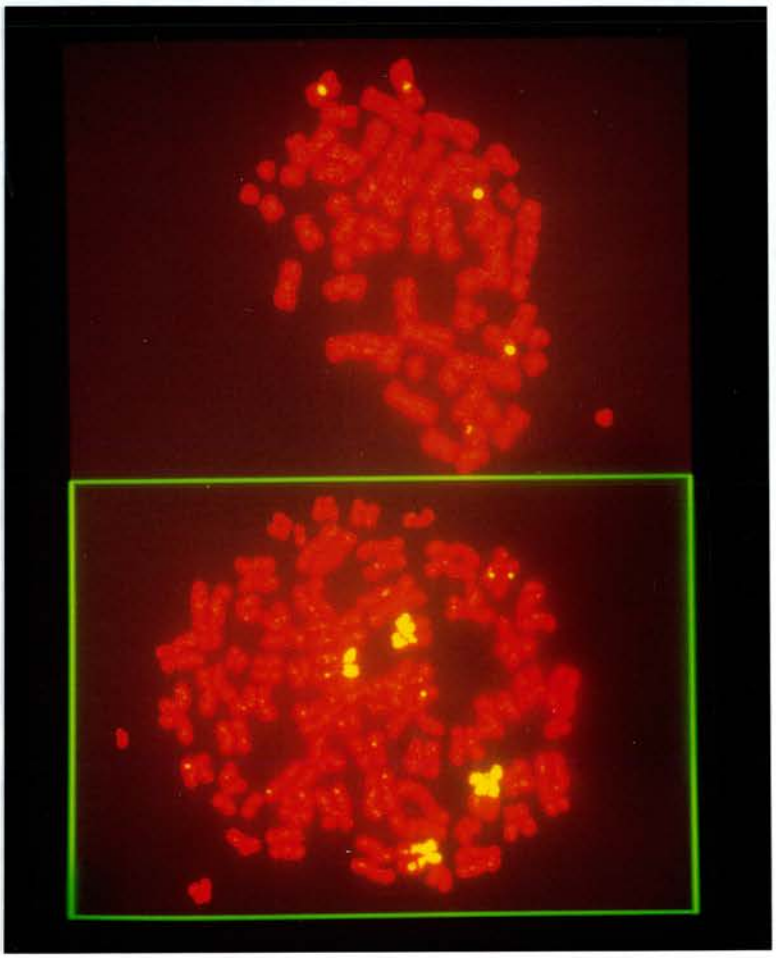



*Top band = neo; lower band = hyg*

*4.5.2.2 In-situ hybridisation*

Formal proof of microcell transfer was obtained by PRINS analysis of the microcell hybrid clones. Figure 4.13 shows PRINS and chromosome 11 paint of OH3, demonstrating that this ovarian cancer cell line has apparently five centromeric regions and that chromosome 11 is markedly rearranged.

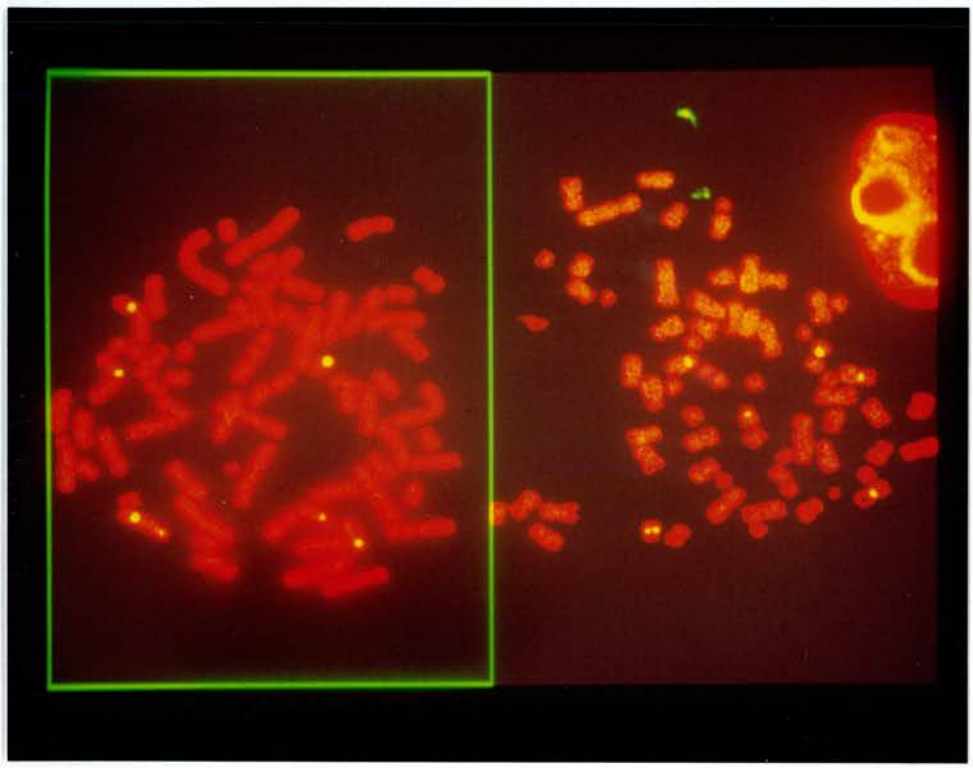
**Figure 4.13 Chromosome 11 primed *in-situ* hybridisation and chromosome painting of OH3**



*PRINS demonstrates 5 chromosome 11 centromeric signals in the recipient (parent) cell line OH3 (top panel). Chromosome painting demonstrates extensive re-arrangement of chromosome 11 in the OH3 parent line.*

Figure 4.14 shows that after chromosome transfer the microcell hybrid now contains 6 centromeres. The example shown was observed over many metaphase spreads for this hybrid.

**Figure 4.14 PRINS demonstrates the transfer of chromosome 11 to OH3**



*Microcell hybrid 11OH2.3 shows six centromeric spots in these two metaphase spreads, representing an increase of one centromeric signal*

*4.5.2.3 Flow cytometric ploidy analysis*

Relative ploidy determination was performed utilising propidium iodide staining and flow cytometric analysis of the microcell hybrids compared with the parent recipient. Table 4.12 presents these results. In summary, OH3 is hypo-tetraploid, and the clones derived from MMCT in the vast majority do not alter the ploidy significantly, confirming that transfer occurs by microcell fusion and not karyoplast/whole cell fusion. At this stage it is pertinent to point out that in the case of 11OH2.4, a non-disjunction event appears to have occurred, with the ploidy being 2N with respect to OH3. This hybrid contained a fragmented transferred chromosome 11 with identical IRS-PCR patterns to 11OH2.1 and 1OH2.2 suggesting that these three clones arose from the same microcell fusion event with a subsequent non-disjunction event accounting for 11OH2.4's ploidy pattern. The identical behaviour of this clone compared with the other chromosome 11 OH3 microcell hybrids in terms of growth suppression (see chapter 5) suggests an absolute growth suppression effect has occurred rather than a relative dosage effect. This is also supported by the observation that OH3 is essentially tetraploid with 5 centromeric signals from chromosome 11; introduction of a single copy of chromosome 11 suppresses growth.

**Table 4.12 Ploidy status of ovarian cancer cell line OH3 and derived microcell hybrids**

| Cells/Cell Line | Ploidy |
|---|---|
| Normal lymphocytes | 2 |
| OH3 | 3.6 |
| 11OH1.1 | 3.8 |
| 11OH2.1 | 3.6 |
| 11OH2.2 | 3.4 |
| 11OH2.3 | 3.5 |
| 11OH2.4 | 6.6 |

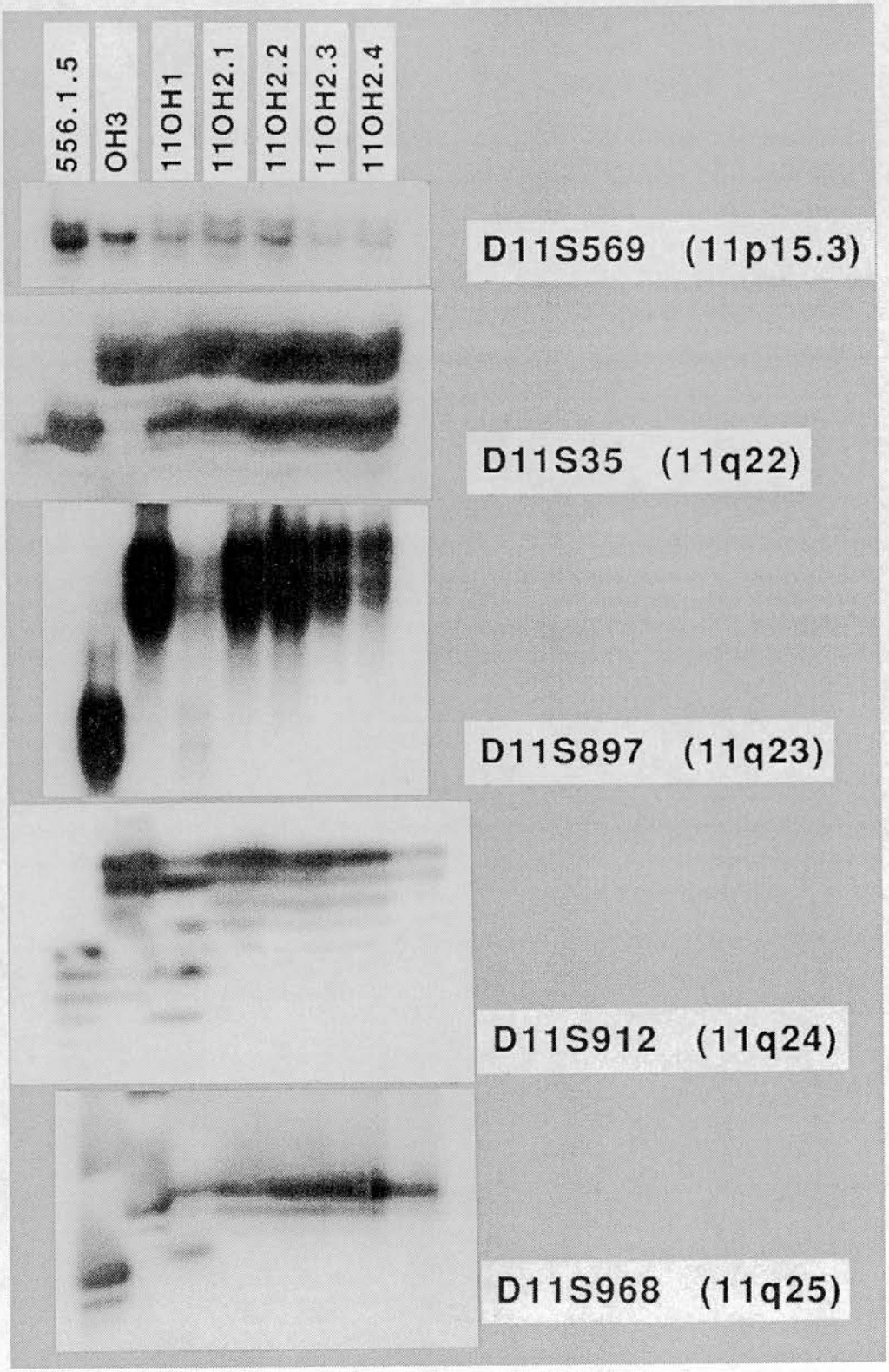
#### *4.5.2.4 Polymorphic microsatellite analysis*

Having confirmed cytogenetically that transfer had occurred, mapping of the transferred chromosome 11 was then performed, initially using the same microsatellites utilised in the LOH and HeLa analyses. In, summary, in contrast to whole chromosome transfer being the rule (as with HeLa), fragmentation of the donor chromosome with several markers not transferred was a common occurrence, and this was in keeping with the disordered status of chromosome 11 in OVCAR3, in contrast with the stable single copy of this chromosome in HeLa. In view of this further markers were analysed using the automated laser fluorescence system at the MRC Human Genetics Unit which allowed microsatellite analysis to be performed more rapidly (see "methods").

#### *4.5.2.5 Autoradiographic analysis*

Using the same markers and methods from the LOH analysis, the OH3 and OHX series of microcell hybrid clones were examined for transfer of chromosome 11 at those loci. Figure 4.15 shows examples of transfer. Note that although microcell hybrid 11OH1.1 has taken up the entire chromosome 11 at all tested loci, hybrids 11OH2.1-2.4 have all failed to take up the region 11q22-qter. These clones were all derived from the same microcell fusion experiment, and provides strong evidence that the clones were all derived from the same fusion event, and were separated from each other at the time of trypsinisation and seeding of the primary fusion plate on to selective media (see "methods"). The interesting finding of partial fragmentation of the chromosome required further analysis with more microsatellite markers and also provided a resource to compare phenotypes of partial versus whole chromosome 11 transfer into OH3.

Figure 4.15 Evidence of transfer of donor chromosome 11 to OH3 by microsatellite analysis



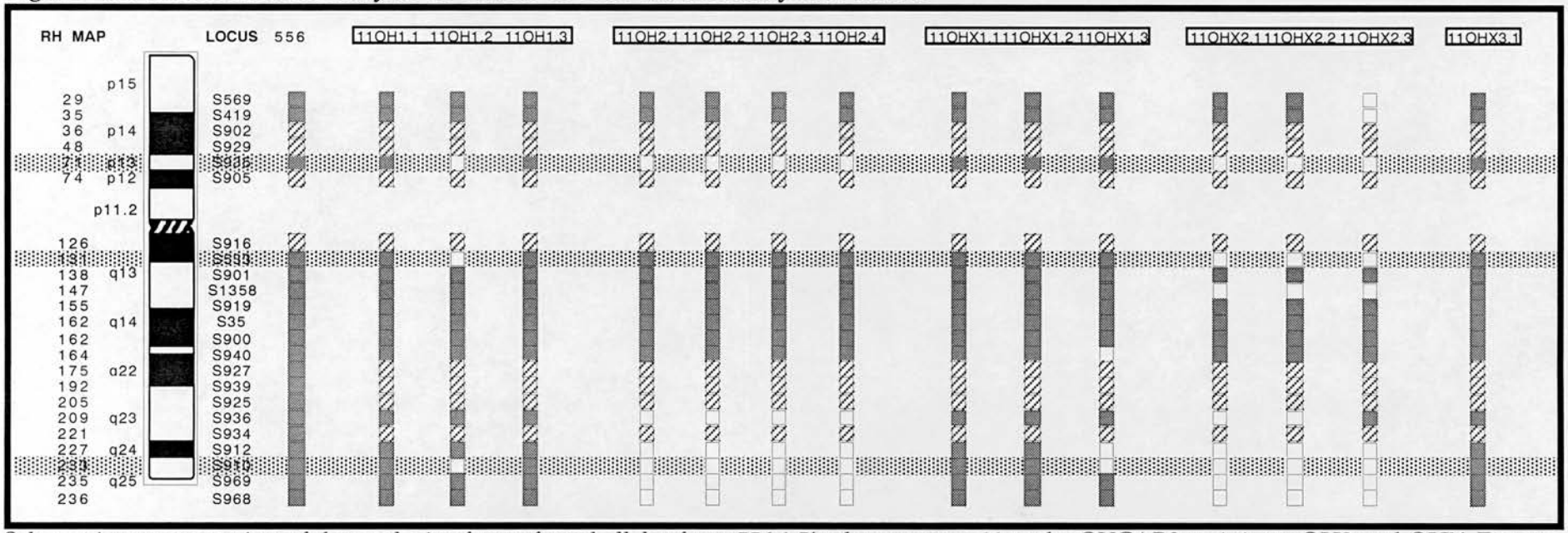
*4.5.2.6 Analysis by automated laser fluorescence*

Extensive further analysis using 23 fluorescently labelled primers was performed. This data is summarised in Figure 4.16. Clones from the same experiment which had identical microsatellite patterns were regarded as identical for the purposes of presenting this data (although they might differ in un-mapped regions). In general there were some differences noted between some clones derived from the same microcell fusion experiment and this is interesting since within an experiment, the IRS-PCR fingerprints were generally identical (attributable to co-transfer of autonomous murine chromatin). This suggested that fragmentation resulting in differences between clones had occurred after the primary fusion event (see below). Some comments require to be made specifically about clones 11OH1, 11OH1.2 and 11OH1.3. These are derived from the same experiment. However, 11OH1.2 and 11OH1.3 are clonal cell lines derived from passage 4 11OH1.1, interesting because it had an initially suppressed phenotype which reverted back to a malignant phenotype indistinguishable from that of the parent OH3. The two clonally derived sub-lines from this passage differ in very few microsatellite markers, yet have totally different phenotypes (see later). Furthermore, the region where they differ includes the 11q24.3-q25 poor-prognosis region defined in the preceding LOH analysis. IRS-PCR patterns are identical between the two sub-clones, and this makes them particularly interesting as potential cloning resources (see "discussion"). The critical region in which these clones differ is shown in Figure 4.17. The ALF tracings are presented for the three microsatellites D11S912, D11S910 and D11S969 from centromeric to telomeric covering a 5 Mb region on 11q24.3-q25. The top panel for each microsatellite shows the donor 556.1.5 allele in green, the recipient OH3 allele in blue, and the hybrid clone 11OH1.1 in purple. The bottom panel shows the derived sub-clones 11OH1.2 in blue and 11OH1.3 in purple. Note that 11OH1.2 has the lost the

donated allele at D11S910 but retained it just 580 Kb telomerically at D11S969 and 4.5 Mb centromerically at D11S912.

The other region demonstrating fragmentational loss in the revertant-phenotype 11OH1.2 clone was D11S935 at 11p13, located in the vicinity of two candidate genes, WT1 and KAI1 (see "introduction" and "discussion").

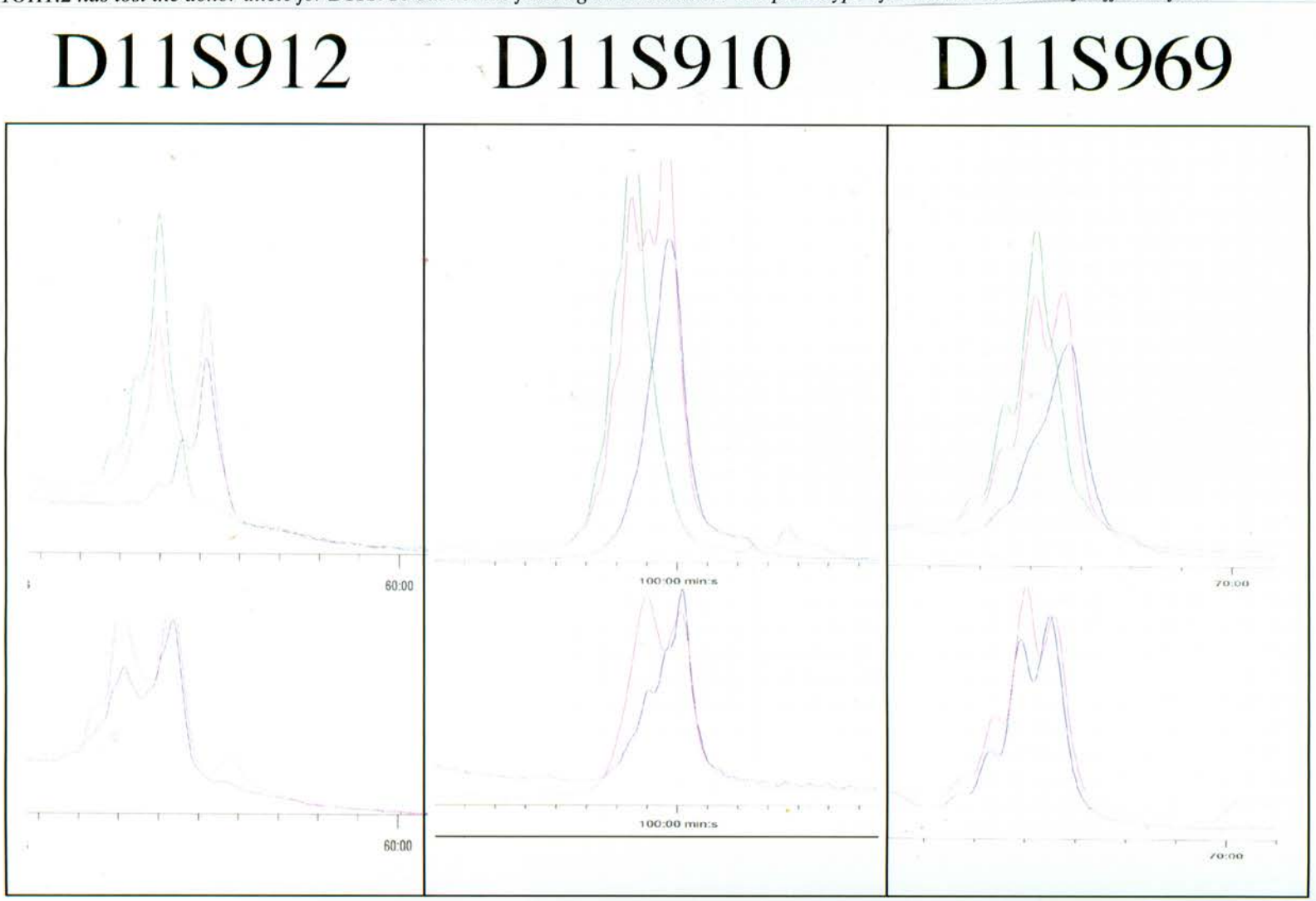
**Figure 4.16 Microsatellite analysis overview of OH3 microcell hybrid clones**




Schematic representation of the analysis of transferred alleles from 556.1.5's chromosome 11 to the OVCAR3 recipients OH3 and OHX. Donor cell line and microcell hybrid clones are shown at *top*. Microsatellite loci used in this analysis are at the *left* of the diagram. Their approximate position is indicated with respect to the chromosome 11 idiogram. The radiation hybrid map position of each of these polymorphic microsatellites is also shown at *left*. *Horizontal shadowed bars* highlight the sites of transferred chromosome disruption associated with functional alteration. ▩, donor allele transferred to recipient hybrid clone. □, donor allele not transferred (therefore chromosome fragmented). ▨, alleles at this locus are identical, therefore locus uninformative.

**Figure 4.17 ALF-PCR analysis of telomeric 11q in 11OH1.1 and derived sub-clones**

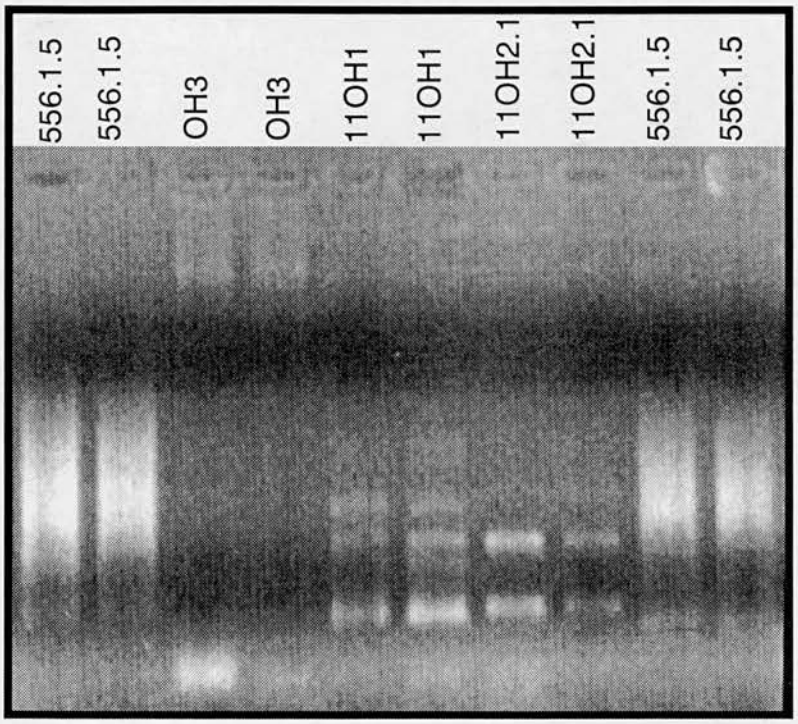
*ALF PCR results are shown for the 3 markers D11S912, D11S910 and D11S969. These markers run from centromeric to telomericat 11q24 (see figure 4.16). In the **top** panel for each marker, 556.1.5 (donor 11) allele is shown in green. Parent OH3 (recipient's chromosome 11) allele is shown in blue and microcell hybrid 11OH1.1 is shown in pink. In the **bottom** panel for each marker, clones 11OH1.2 and 11OH1.3 are shown in blue and pink respectively. These are clonal lines derived from 11OH1.1. As can be seen 11OH1.2 has lost the donor allele for D11S910 but not the flanking microsatellites. The phenotype of 11OH1.2 is radically different from 11OH1.3 (see chapter 5)*



*4.5.2.7 Interspersed repetitive sequence (IRS) PCR: co-transfer of murine sequences.*

IRS-PCR using L1/B1 primers to produce a murine fingerprint of the hybrid clones was performed. Figure 4.18 shows L1/B1 IRS-PCR fingerprint examples for the OH3 microcell hybrid clones.

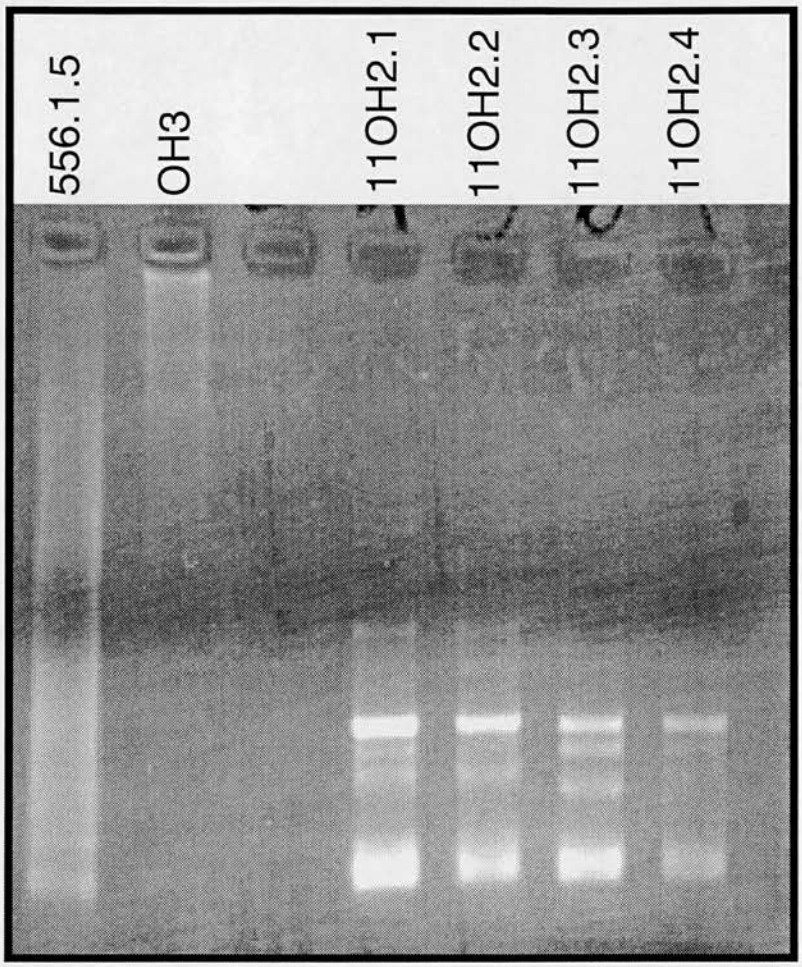
**Figure 4.18 L1/B1 IRS-PCR comparison of hybrids 11OH1.1 and 11OH2.1**




As can be seen, the murine fingerprints differ between experiments 11OH1 and 11OH2.

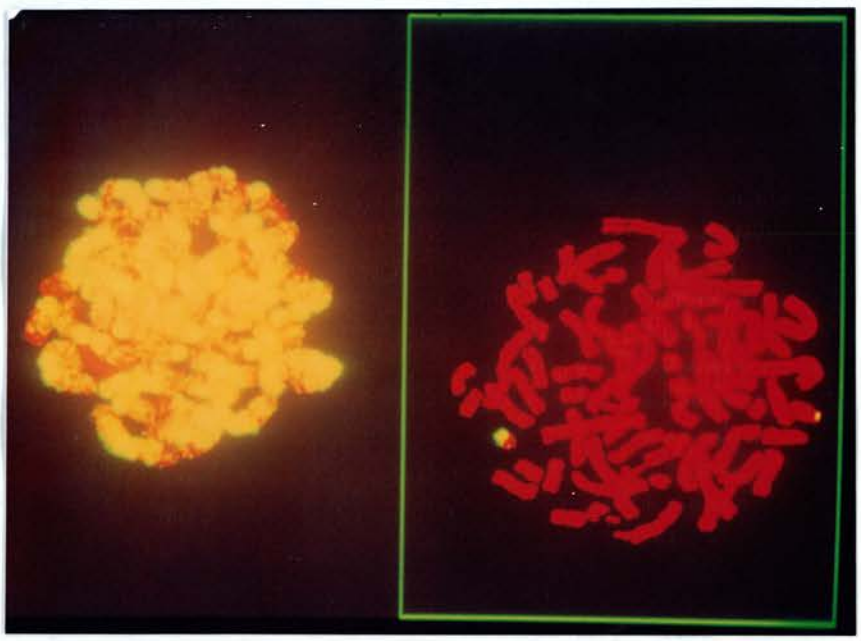
However, within an experiment, the IRS-PCR fingerprints were generally identical. Figure 4.19 shows that IRS-PCR is identical for 11OH2.1, 2.2 and 2.4. It is, however, slightly different for 11OH2.3

**Figure 4.19 L1/B1 IRS-PCR fingerprints for 11OH2 microcell hybrids**




*In-situ* hybridisation using mouse cot-1 DNA showed that the IRS-PCR fingerprint for 11OH2.1 was associated with tiny co-transferred murine chromosome fragments (see Figure 4.20)

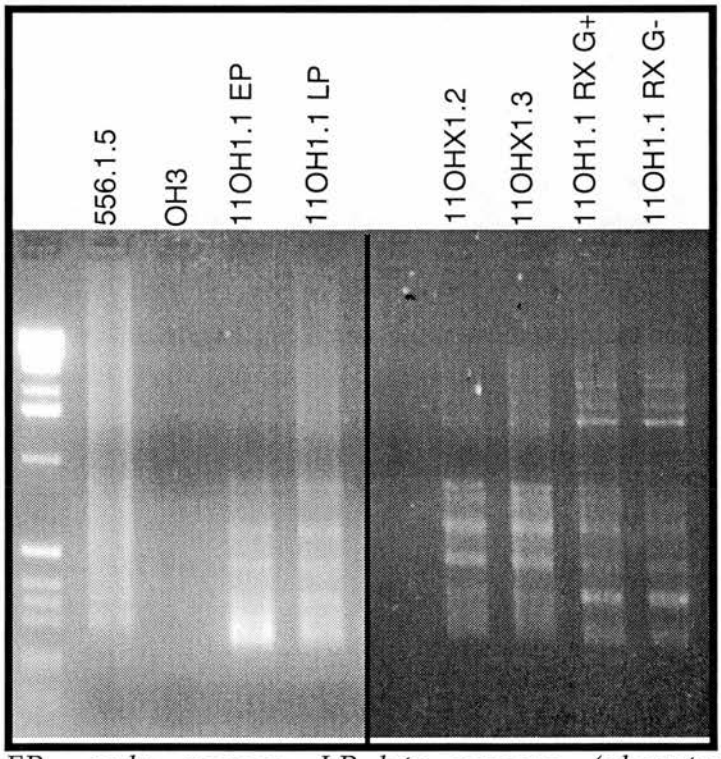
**Figure 4.20 Mouse cot-1 chromosome paint for 556.1.5 and hybrid 11OH2.1**



*Left panel shows mouse cot-1 paint of the murine donor line 556.1.5. Right panel shows microcell hybrid 11OH2.1 containing a fragment of murine chromosome demonstrated by mouse cot-1 painting*

For 11OH1.1, which had retained a whole copy of chromosome 11 initially, two derived sub-clones were isolated with markedly different phenotypes. These phenotypic differences were associated with differences in the retained regions of chromosome 11 (see above). However, it could also have been possible that the suppressed phenotype was generated by co-transferred murine chromatin, and therefore phenotypic reversion to the malignant parent state could have resulted from loss of that suppressor murine sequence since it was not dominantly selected for. Gratifyingly, IRS-PCR confirms co-identical murine fingerprints for 11OH1.1 and the derived sub-clones 11OH1.2 and 11OH1.3, suggesting that it is indeed chromosome 11 alterations that are responsible for the altered phenotype (see Figure 4.21).

**Figure 4.21 L1/B1 IRS-PCR for 11OH1.1 and derived sub-clones**




*EP= early passage LP=late passage (phenotypic reversion noted) 11OH1.1RXG- = 11OH1.1 grown as a xenograft(X) in a SCID mouse and recovered (R)as a cell line in G418 free media (G-). 11OH1.1RXG+ = 11OH1.1 grown as a xenograft and recovered as a cell line in G418 containing media. Background mouse cells from xenograft are deleted by concurrent use of hygromycin in both media.*

# 5. FUNCTIONAL ANALYSIS OF MICROCELL HYBRIDS

## 5.1 HeLa chromosome 11 microcell hybrids

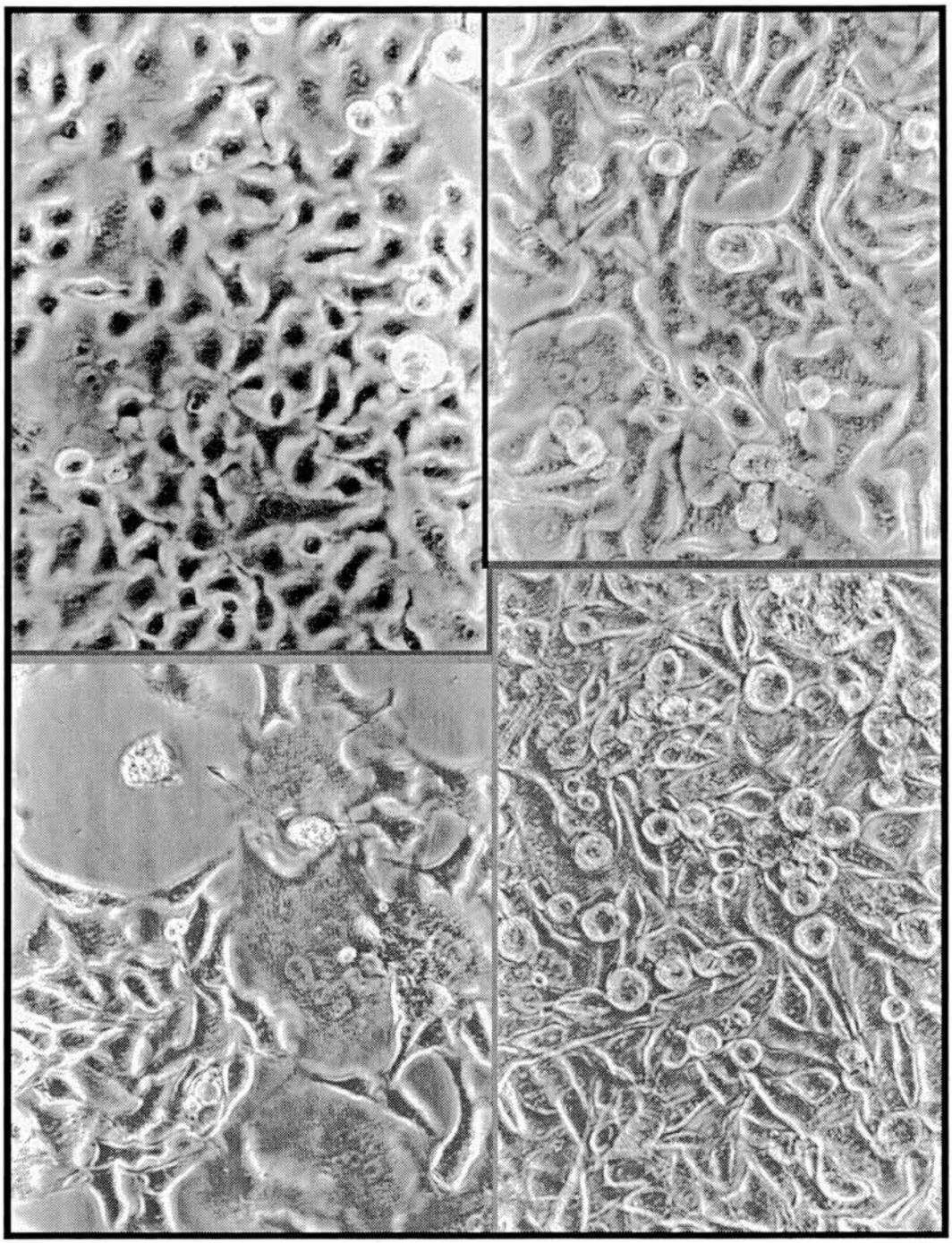
Confirmation that whole chromosome 11 had been transferred to the primary microcell hybrid clones afforded the opportunity to repeat Saxon's experiment of 1986 (Saxon et al, 1986) which provided the first evidence of functional suppression of tumorigenicity using MMCT.

### 5.1.1 Morphology

All chr. 11/HeLa microcell hybrid clones had the same morphological characteristics as the parent HeLa Ohio cell line. One clone however became progressively larger and the cells senesced. However, a minority of revertant clones escaped senescence and grew rapidly with the same characteristics as the parent HeLa line. Figure 5.1 shows the sequence of events associated with clone 11H1.1.

The top left plate shows parent HeLa cells. Top right shows microcell hybrid 11H1.1, with enlarged cells. Bottom left shows progressively enlarging, senescing cells, and a revertant colony at 7 o'clock that has escaped senescence. These revertant clones morphology identical to the HeLa parent were expanded into cell lines for further analysis (bottom right). Since the chr.11/HeLa microcell experiments were not the final objective of this work, they were not functionally analysed in great detail, however, xenograft analysis was performed to repeat Saxon's observation of suppression of tumorigenicity by the introduction of chromosome 11 into HeLa.

**Figure 5.1 Photomicrographs of HeLa and microcell hybrids**



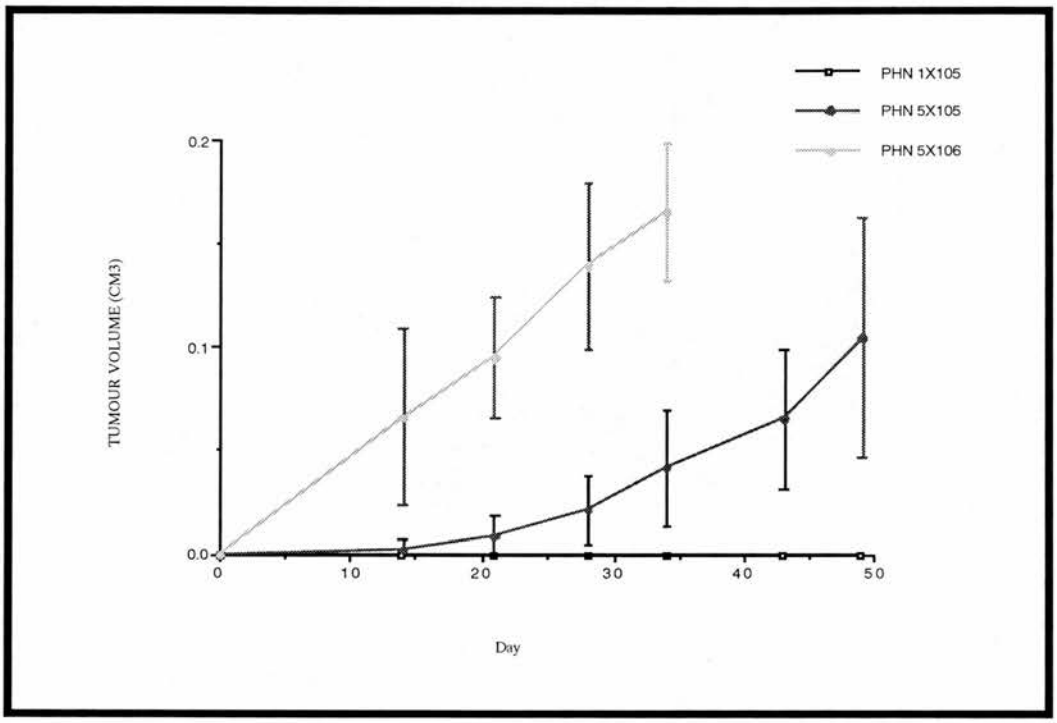
*HeLa parent cell line (top left); microcell hybrids 11H1.1 (top right); revertant colony emerging from background of senescing cells (bottom left); expansion of revertant colony r1 as a cell line (bottom right).*

### 5.1.2 Xenograft analysis of HeLa and HeLa microcell hybrids.

Initially, the tumorigenicity of the HeLa parent was tested to provide a guide for the required xenograft conditions to directly compare the parent HeLa with the microcell hybrids.

Figure 5.2 shows titration of cell numbers versus xenograft growth for the *neo*-transfected HeLa control cell line PHN in nude mice.

**Figure 5.2 Growth of control HeLa cell line PHN in nude mice**

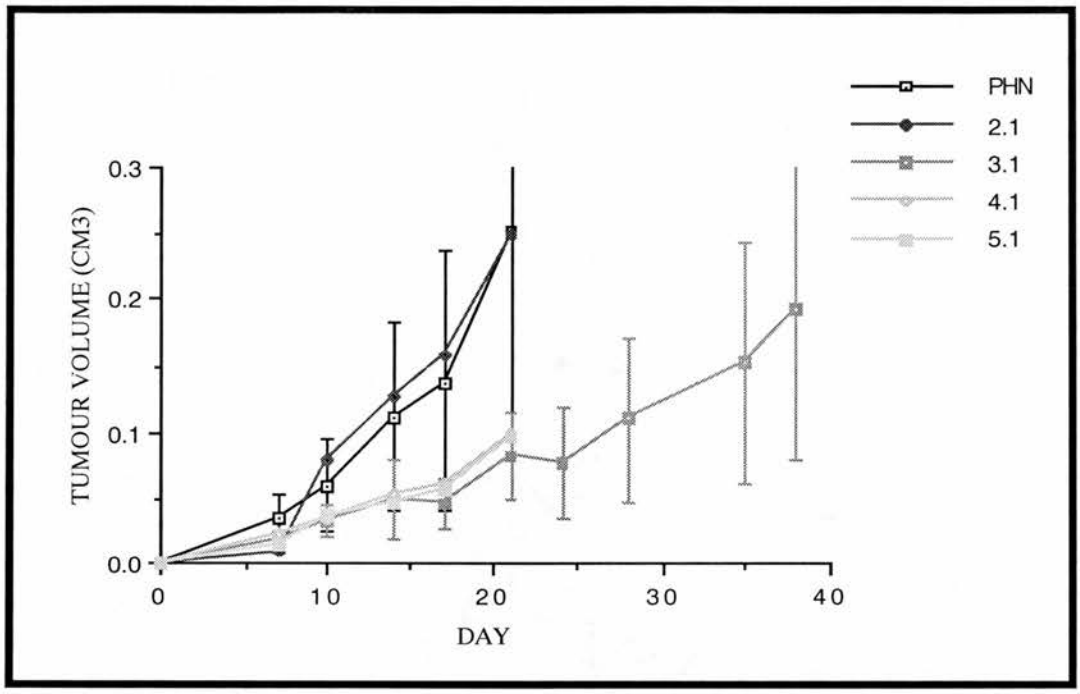



*Cell numbers injected are shown in the legend at top right. Mean values for 4 tumours. Error bars represent standard deviation.*

As can be seen, tumour growth did not occur at 1 X $10^5$ cells per injection but did occur at 5 X $10^5$ cells per injection or more.

Several microcell hybrid clones were then tested in direct comparison to the *neo*-transfected HeLa line PHN. This data is presented in Figure 5.3.

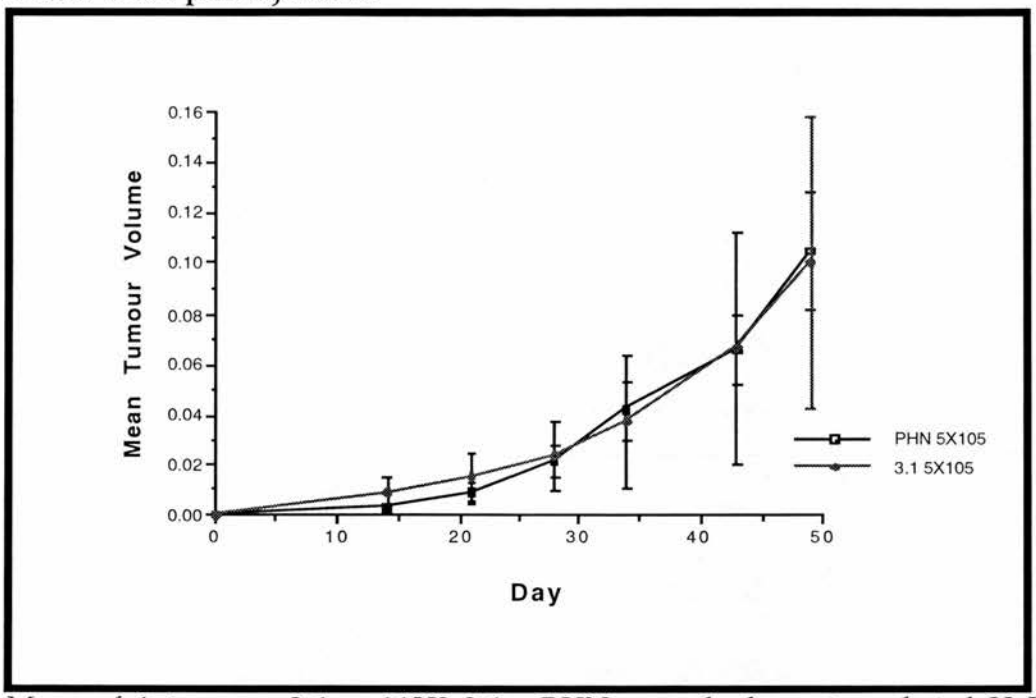
**Figure 5.3 Direct comparison of PHN control HeLa cell line with HeLa microcell hybrid (11H2 series)**




*Each point represents the mean of 4 tumours, with standard deviation bars shown. 5 X $10^5$ cells were used per injection.*

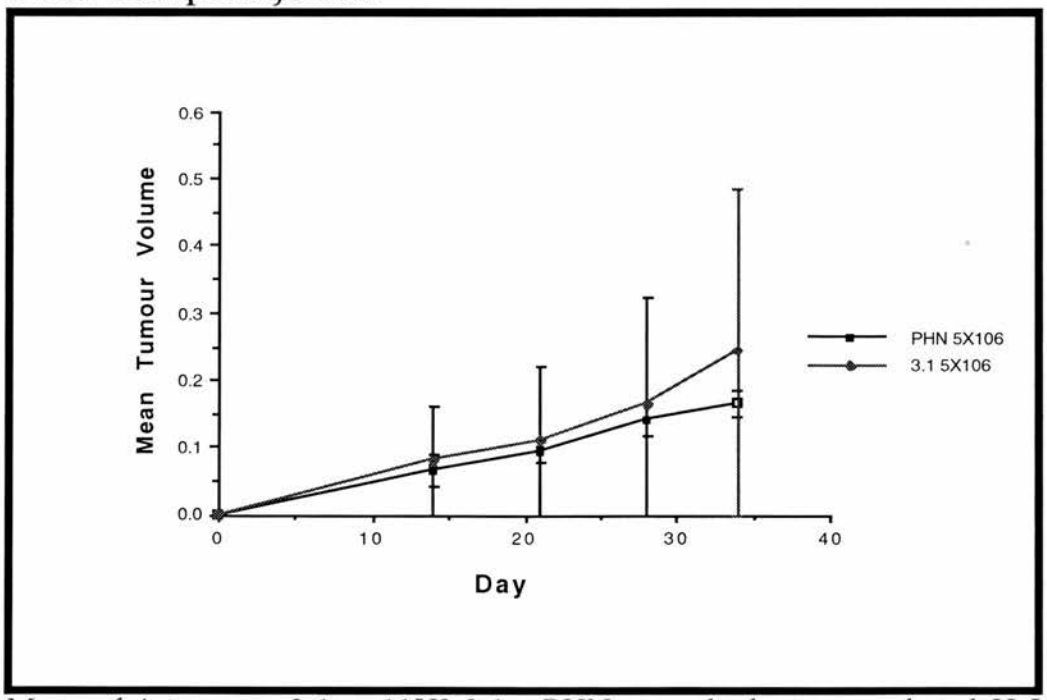
As can be seen from the above figure, no suppression of tumorigenicity was observed, a surprising finding given Saxon's original report. However, apparent growth delay was observed for some clones, and this was most pronounced for microcell hybrid 11H3.1. Xenograft experiments were repeated with this cell line, compared directly with PHN at 5 X $10^5$ cells per injection (Figure 5.4) and also at 5 X $10^6$ cells per injection (Figure 5.5). As can be seen from both of these experiments, there was no difference in growth rate between 11H2.3.1 and PHN suggesting no growth delay. This raises many issues about the discrepancy with Saxon's original observations (see discussion).

**Figure 5.4 Comparison of 11H2.3.1 hybrid clone with PHN control: 5 X $10^5$ cells per injection**




*Mean of 4 tumours.3.1 = 11H2.3.1 . PHN = pool of neo transfected HeLa clones*

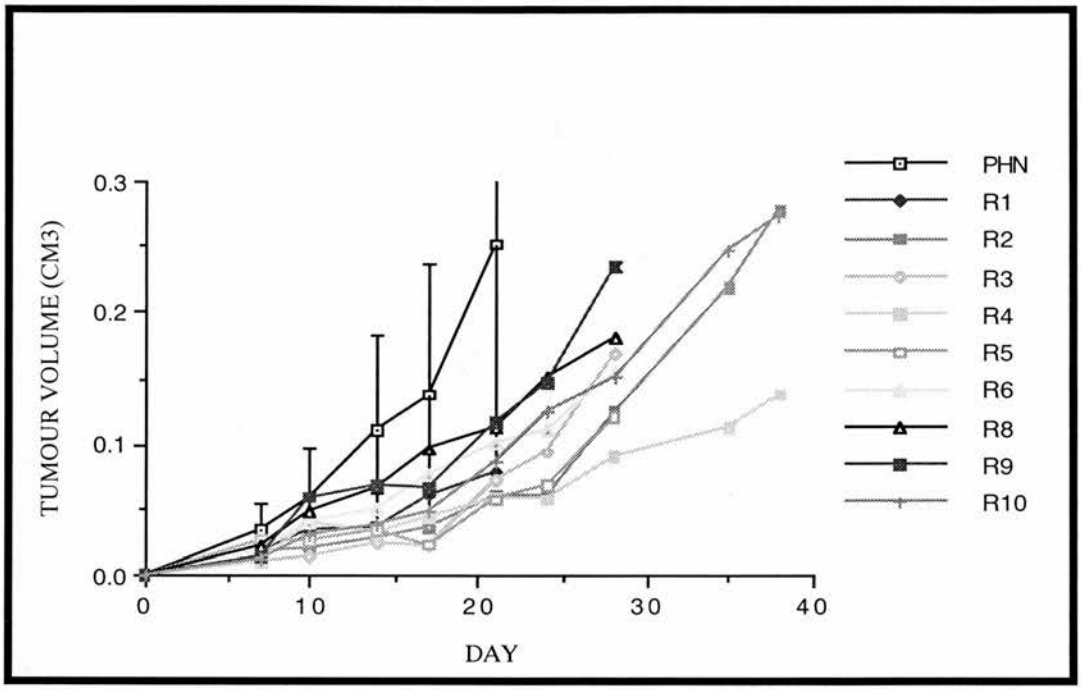
**Figure 5.5 Comparison of 11H2.3.1 hybrid clone with PHN control: 5 X $10^6$ cells per injection**




*Mean of 4 tumours.3.1 = 11H2.3.1 . PHN = pool of neo transfected HeLa clones*

Several clonal revertant cell lines derived from 11H1.1 (see Figure 5.1) were also tested against PHN as xenografts. Figure 5.6 presents this data, In summary, there was an apparent growth delay effect, but this was not statistically significant.

**Figure 5.6 Comparison of tumorigenicity of clonal revertants derived from 11H1.1 with HeLa control PHN**




*Clonal revertants R1-R10 all derived from 11H1.1*

## 5.2 OVCAR3 chromosome 11 microcell hybrids

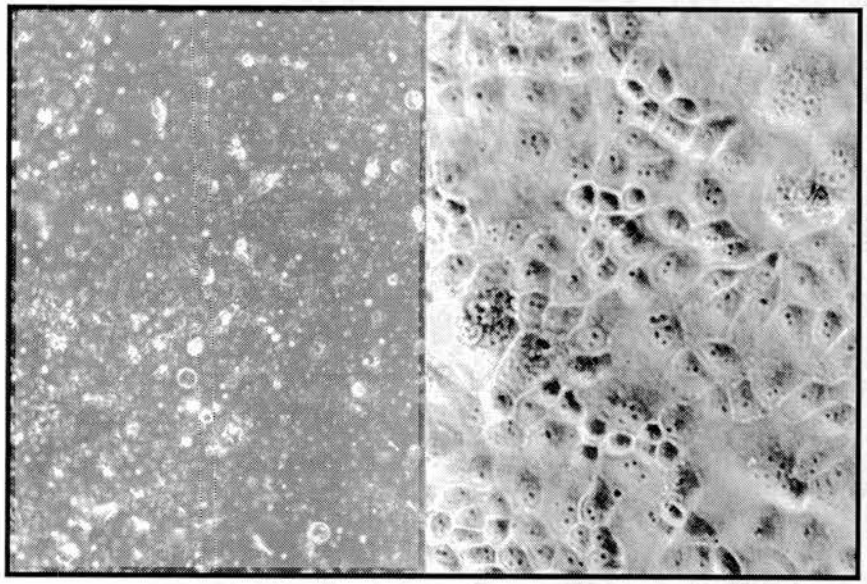
### 5.2.1 Morphology of hybrids

The immortalised phenotype of the parent was retained in the microcell hybrids, and with the exception of 11OH1.1/1.3 and 11OHX1.1, the morphology of the cells was identical to the parent OH3. Altered morphology was observed in the aforementioned lines, most strikingly with 11OH1.3. The cells remained rounded, closely packed and invisibly "corralled", though cell numbers were not noticeably fewer than other chromosome 11 hybrids. This suggested a defect in cell spreading or motility, and provided an interesting parallel observation to the associations between 11q24 LOH and advanced stage/ poor survival from the clinical specimens. Photomicrographs of different clones from the 11OH series are presented in Figure 5.7

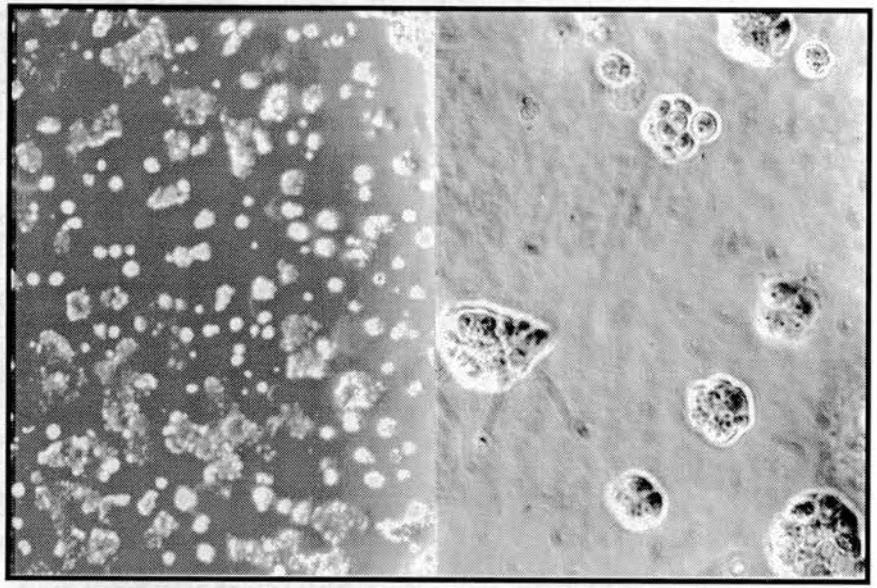
**Figure 5.7 Morphology of OVCAR3 and chromosome 11 microcell hybrids**

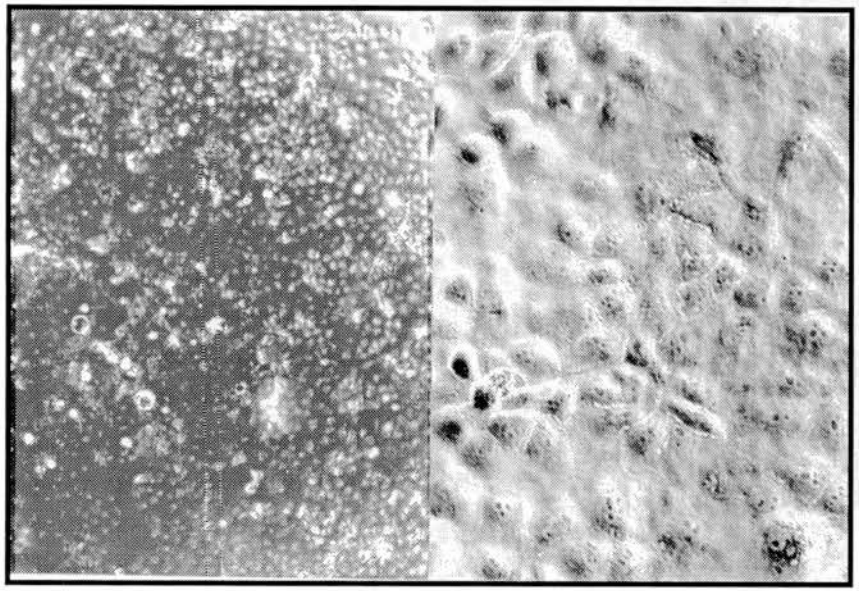
*Despite differing growth rates, OHN, 11OH1.2 and 11OH2.4 have very similar morphology in-vitro. In contrast, 11OH1.3 has completely different morphological characteristics, the tendency to spread out on tissue culture plastic appears to be abrogated*

OHN (Parent control)

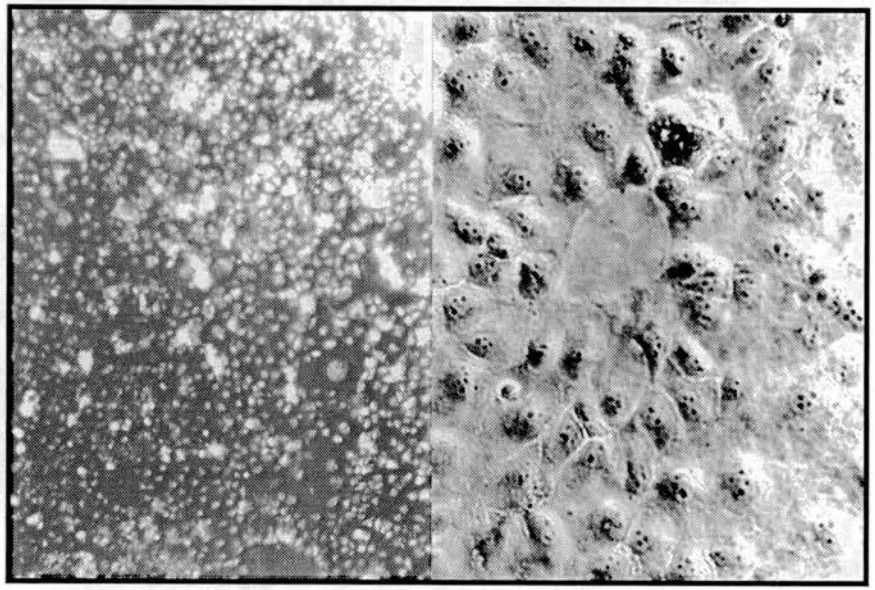


11OH1.3 ( Whole 11 transfer)





11OH1.2 (del 11q24 / 11p13)



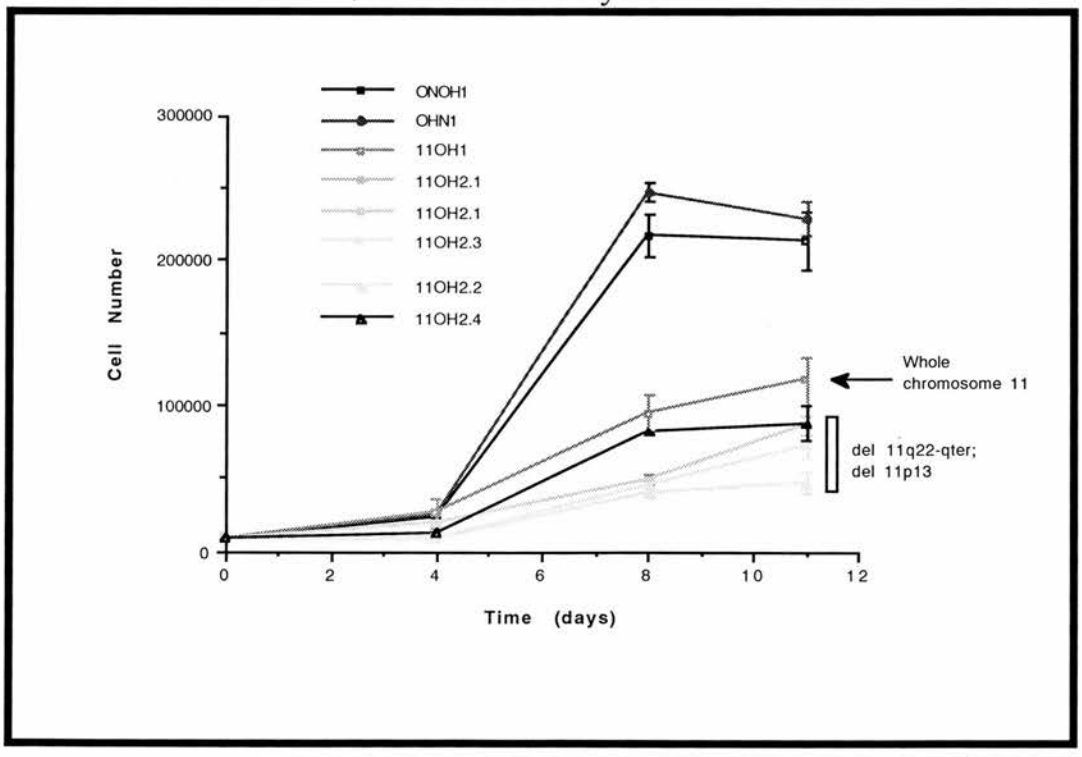
11OH2.4 (del 11q22-qter / 11p13)

### 5.2.2 *In-vitro* growth of hybrids

#### *5.2.2.1 11OH microcell hybrid series*

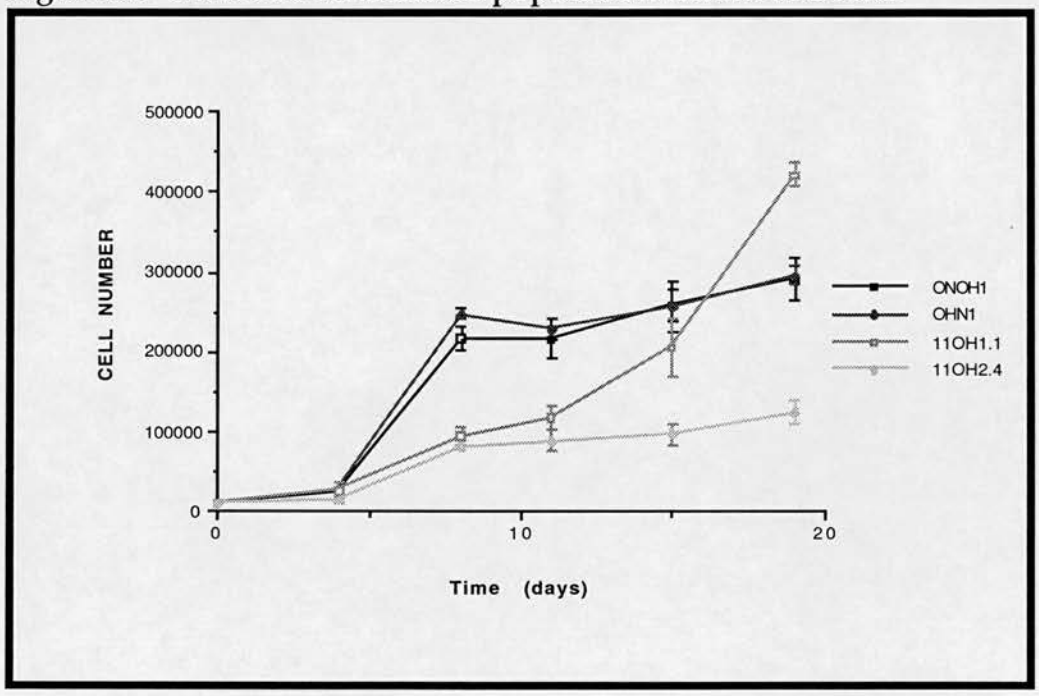
Five clonal hybrid cell lines were derived from microcell fusion experiments 11OH1 and 11OH2. Clone 11OH1.1 contained a complete transferred chromosome 11. The four clones from the 11OH2 fusion (11OH2.1-2.4) were identical in terms of transferred microsatellite markers (with deletion of 11q22-qter and 11p13, see figure 4.16) of the donated chromosome and also by murine L1/B1 IRS-PCR fingerprinting (figure 4.19). Figure 5.8 shows *in-vitro* growth of these clones in a typical experiment with quadruplicate samples with 1 X $10^4$ cells initially seeded. This experiment was repeated three times and the result was similar in each case. In summary, the 11OH1 and 11OH2 series of clones were equally growth suppressed, suggesting that a growth suppressor exists out-with 11p13 and 11q22-qter.

**Figure 5.8 *In-vitro* growth of control OH3 lines and chr 11/OH3microcell hybrids**



However, prolonged growth showed that 11OH1.1 grew rapidly at higher confluence with a clear bi-phasic pattern suggesting that clonal divergence had occurred with a sub-population which rapidly overgrew the original clone (see Figure 5.9)

**Figure 5.9 Evidence for a mixed population within 11OH1.1**




Clonal sub-lines were derived by single cell cloning of 11OH1.1 at passage 4 which had clearly different phenotypes. Microsatellite analysis of these showed deletion of a 5.5 Mb interval on 11q24.3-q25 (see figure 4.20) despite identical IRS-PCR fingerprints. It is almost certain that the phenotypic differences attributed to these clones are due to fragmentation of the donated chromosome 11. The alternative explanation is that the observed phenotypes are the result of clonal selection. However the original recipient ovarian cancer line was clonally derived by *hyg*-plasmid transfection and the phenotypic divergence of 11OH1 was noted within four passages of the derivation of the clonal microcell hybrid line. These factors make it highly unlikely that these effects are simply due to clonal selection. It is possible that the phenotypic differences are due to clonal divergence by

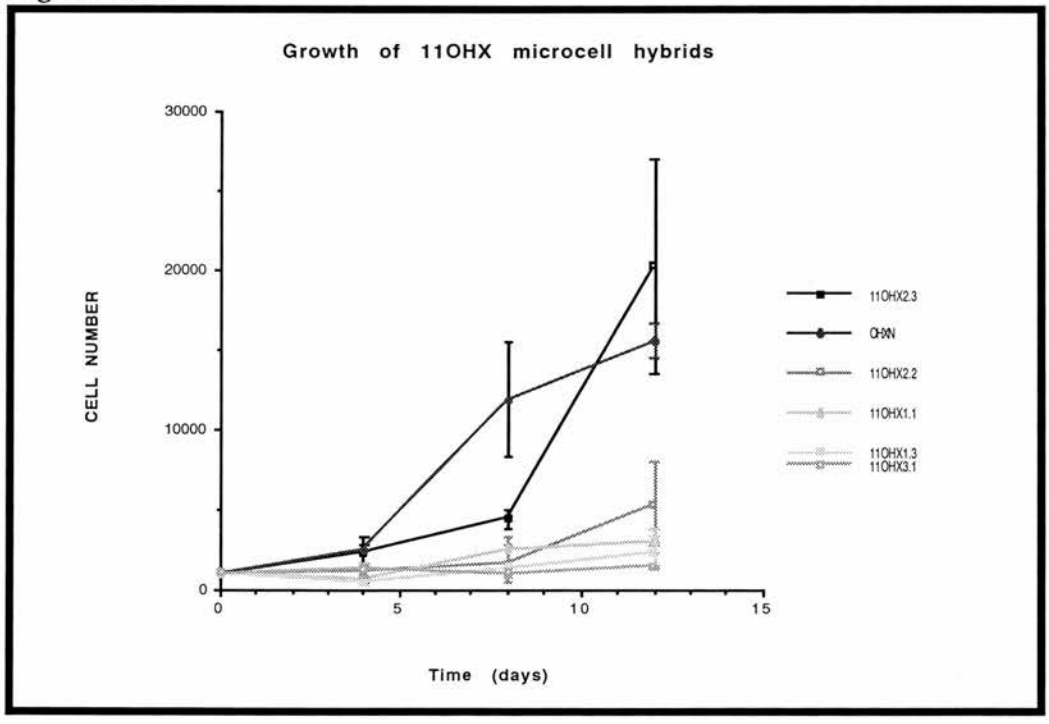
mutation at another (non-chromosome 11) locus. This possibility cannot be formally excluded, however, the fact that the murine IRS-PCR fingerprints are identical for 11OH1.1, 1.2, and 1.3 strongly suggests that other loci are not involved.

### *5.2.2.2 The 11OHX microcell hybrid series.*

Due to the difficulties in establishing convenient xenografting conditions with OH3 (see below), a single xenograft that grew successfully was rescued back and the OHX cell line was established from it. This cell line much more readily formed tumours in SCID mice. In view of this three further microcell fusions were carried out transferring chromosome 11 to the OHX recipient ovarian cancer cell line. Clones from two fusion experiments received whole chromosome 11 (11OHX1 and 11OHX3) and partial transfer was achieved in a further experiment (11OHX2) (see Figure 4.16).

Figure 5.10 shows comparison of these clones, seeding 1000 cells initially.

**Figure 5.10**

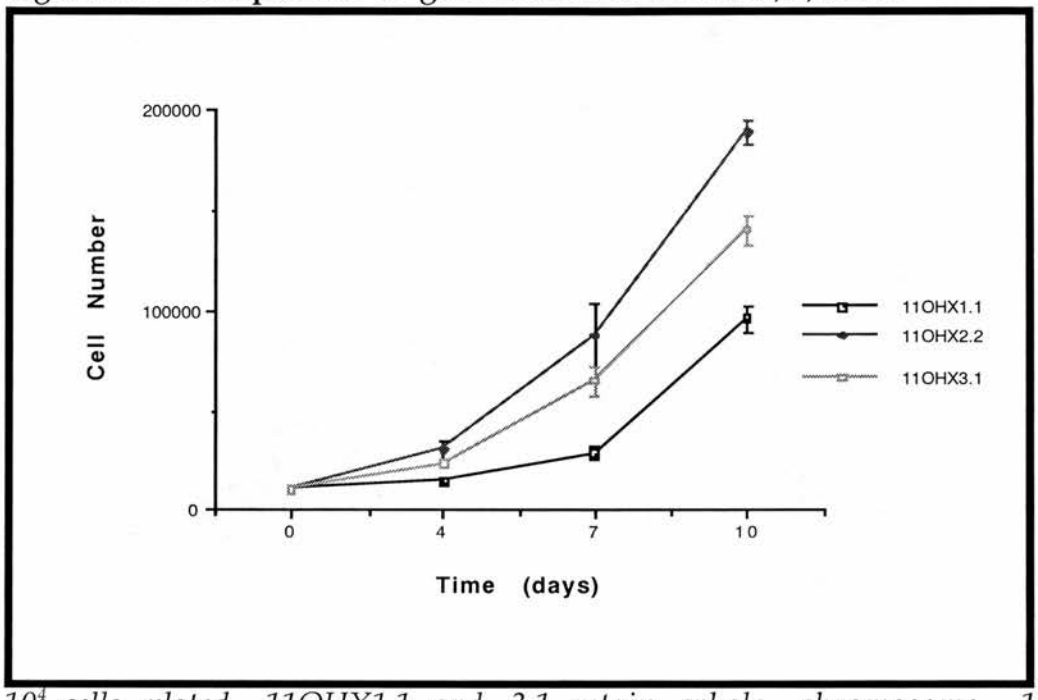



*Transferred chromosome status is: 11OHX1 (whole 11), 11OHX2 (del 11q22-qter; del 11p13), 11OHX3 (whole 11)*

11OHX2.3 grows with the same characteristics as the *neo*-transfected control cell line. By day 12, 11OHX2.2 is starting to diverge.

In another experiment, using $10^4$ cells initially, the 11OH2 series clearly demonstrates faster relative growth compared with 11OHX1 or 11OHX3 (see figure 5.11).

**Figure 5.11 Comparison of growth rates of 11OHX1, 2, and 3**




*$10^4$ cells plated. 11OHX1.1 and 3.1 retain whole chromosome 11. 11OHX2.2 has deletions of 11q22 and 11p13.*

In order to address the reasons for the observed growth suppression for chromosome 11 microcell hybrids seen in figures 5.8 and 5.9, the possibilities were considered that such growth inhibition was due to cell-cycle block caused by the introduction of a cell-cycle regulator, or that apparent growth inhibition was in fact due to increased apoptosis.

### 5.2.3 Cell-cycle distribution of hybrids by flow cytometry

Analysis of the cell cycle was performed on two separate occasions for each hybrid line grown to mid-log phase simultaneously as a group. Flow cytometric (FACS) cell-cycle analysis of propidium iodide stained cells was performed. In summary, there was no significant difference in any of the microcell hybrids compared with the control in terms of

cell-cycle distribution. This information is summarised in Tables 5.1 and 5.2 for the 11OH series and in Table 5.3 for he 11OHX series

**Table 5.1 Cell-cycle analysis of 11OH series microcell hybrids**

| | OH3 | 5OH3.1 | 5OH3.2.1 | 5OH3.2.2 | 5OH3.2.3 |
|---|---|---|---|---|---|
| G1 | 57% | 43% | 39% | 43% | 42% |
| S | 29% | 30% | 27% | 35% | 28% |
| G2+M | 14% | 27% | 34% | 23% | 31% |
| PROLIFERATIVE INDEX | 43% | 58% | 61% | 58% | 59% |
| PLOIDY | 1.80 | 1.89 | 1.78 | 1.72 | 1.74 |

**Table 5.2 2nd analysis of 11OH series microcell hybrids**

| | COLO 205 | ONOH | OHN | 11OH1.2 | 11OH1.3 | 11OH2.2 | 11OH2.4 |
|---|---|---|---|---|---|---|---|
| G1 | 85% | 64% | 69% | 67% | 64% | 57% | 75% |
| S | 5% | 29% | 17% | 21% | 18% | 28% | 18% |
| G2+M | 10% | 8% | 14% | 12% | 18% | 16% | 7% |
| PROLIFERATIVE INDEX | 15% | 38% | 31% | 33% | 36% | 44% | 25% |
| PLOIDY | 1.71 | 3.50 | 1.84 | 2.83 | 1.89 | 1.71 | 3.34 |

*Colo205= readily apoptotic colorectal cancer cell line*

The 11OHX series also did not show evidence of alteration of cell-cycle distribution.

**Table 5.3 Cell-cycle analysis of the 11OHX microcell hybrids**

| | OHXN | OHX | 11OHX1.1 | 11OHX1.3 | 11OHX2.2 | 11OHX2.3 | 11OHX3.1 |
|---|---|---|---|---|---|---|---|
| G1 | 52% | 63% | 62% | 62% | 55% | 58% | 58% |
| S | 27% | 26% | 20% | 23% | 26% | 26% | 22% |
| G2+M | 21% | 12% | 18% | 15% | 19% | 16% | 20% |
| PROLIFERATIVE INDEX | 48% | 38% | 38% | 38% | 45% | 42% | 42% |
| PLOIDY | 1.61 | 1.83 | 1.7 | 1.75 | 1.66 | 1.58 | 1.66 |

### 5.2.4 Apoptosis analysis of hybrids by Annexin V flow-cytometry and the FluoroTUNEL assay

The possibility was also considered that apoptosis (programmed cell death) might be responsible for the observed growth inhibition of the chromosome 11 microcell hybrids. Using FITC labelled Annexin V in a flow-cytometric analysis (Vermes et al, 1995), the population corresponding to "early apoptosis" (Annexin V positive, propidium iodide negative) were identified. In summary, the microcell hybrid clones did not exhibit increased apoptosis under normal conditions of cell growth to explain their impaired growth rate (see Table 5.4).

**Table 5.4 Annexin V-FACS analysis of 11OH hybrid series**

| Cell Line | % Apoptosis (Annexin V +; PI -) |
|---|---|
| Colo 205 (positive control) | 23% |
| OHN (parent) | 2.7% |
| 11OH1.1 | 4.7% |
| 11OH1.2 | 3% |
| 11OH1.3 | 4% |
| 11OH2.3 | 4.4% |
| 11OH2.4 | 5% |

As can be seen, no induction of apoptosis is associated with transfer of chromosome 11 into OH3. This analysis was repeated twice with almost identical results. Additionally, this finding was confirmed using the FluoroTUNEL technique, with no evidence of induction of apoptosis for the microcell hybrid under conditions of normal cell growth relative to the percentage of apoptotic cells found in the parent cell lines.

The same analysis was applied to the 11OHX series of hybrids. OHX had a higher apoptosis score but again, no induction of apoptosis was apparent (Table 5.5).

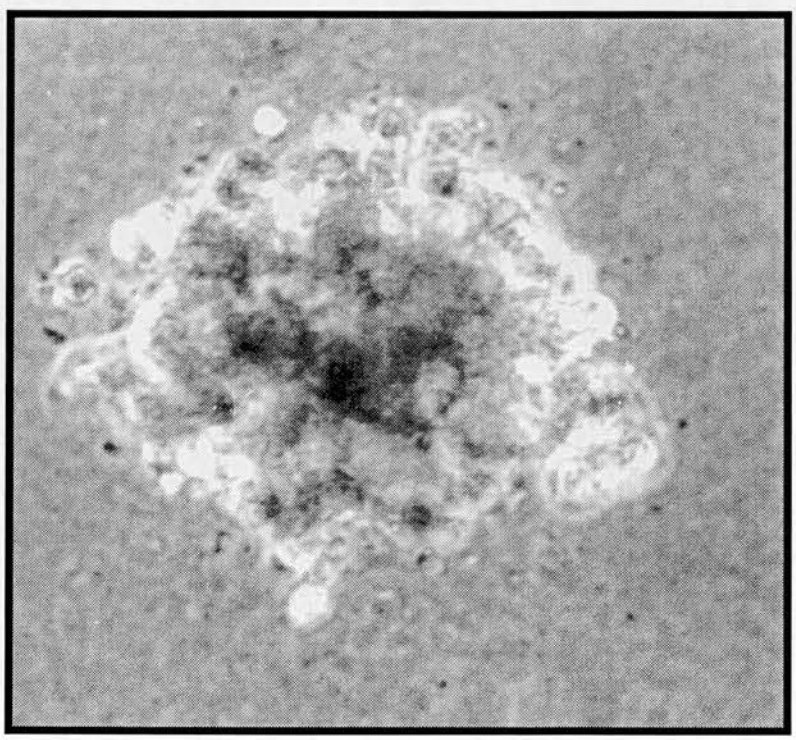
Table 5.5 Annexin V-FACS analysis of 11OHX hybrid series

| Cell Line | % Apoptosis (Annexin V +; PI -) |
|---|---|
| Colo 205 | 22.6% |
| OHX | 21.7% |
| OHXN | 20.6% |
| 11OHX1.1 | 19.7% |
| 11OHX1.3 | 21.5% |
| 11OHX2.2 | 10% |
| 11OHX3.1 | 22.3 |

### 5.2.5 Soft-agar clonogenicity of hybrids

The ability of cancer cells to grow in soft agar, does represent a measure of their tumorigenic potential *in*-vivo although the correlation is by no means strict. The soft agar clonogenic assay was employed to assess differences in the clonogenic potential of the microcell hybrids. Given the lack of ease of xenograft tumour formation, it was not surprising that clonogenicity was fairly low in the parent cell line. An example of an OH3 soft agar clone is shown in Figure 5.12

**Figure 5.12 OH3 clone growing in soft agar**



The results of the soft agar assay are presented in Table 5.6, and in summary show no alteration in the microcell hybrids of the already poor clonogenic potential of the parent cell line.

**Table 5.6 Soft agar clonogenicity of OH3 and hybrids**

| | Mean | Std Deviation |
|---|---|---|
| ONOH | 25.5 | 2.9 |
| 11OH1 | 22.2 | 2.6 |
| 11OH2.2 | 27 | 3.5 |
| 11OH2.3 | 25 | 6.6 |
| 11OH2.4 | 21.2 | 2.9 |

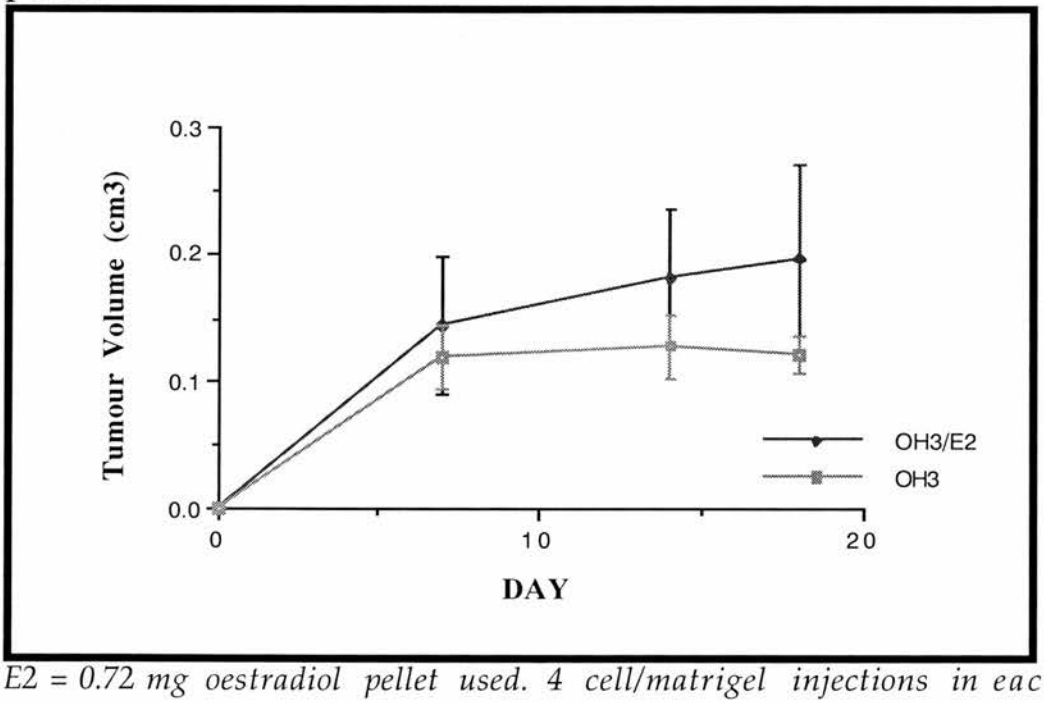
*Mean derived from 4 plates; colonies per $10^5$ cells seeded*

### 5.2.6 Tumorigenicity of xenografted hybrids

Difficulties were encountered in trying to optimise conditions for routine xenograft formation. Cell numbers up to 4 X $10^7$ per injection failed to produce xenografts in nude mice in more than 2/6 injections on three occasions using OH3, derived from the tumorigenic OVCAR3. Since microcell hybrids had already been produced, it was imperative that the conditions for routinely successful xenografting were determined for OH3. Matrigel, a commercially available basement membrane preparation derived from the Engelbreth-Holm-Swarm murine tumour (Kleinman et al, 1982) was used to stimulate xenograft formation, but no growth was observed in nude mice. The decision was then taken to change to using SCID (severe combined immunodeficiency) mice, since these had no humoral or cell mediated immune response, although they had the disadvantage of being furry (more difficult to accurately and precisely measure the tumours). The take rate using matrigel was substantially improved in SCID mice. OVCAR3 is known to be ER and PR positive, and that oestradiol can moderately increase the cell line's growth rate

(Hamilton et al, 1983). In view of this, low dose oestradiol pellets (0.72 mg) were implanted sub-cutaneously at a site distant from the injection sites, and this resulted in a moderate increase in the tumours' growth rates but more importantly, a more consistent take rate approaching 100% with $10^7$ cells per injection. Figure 5.13 shows the effect implanting oestradiol sustained release pellets (0.72 mg) sub-cutaneously in SCID mice and injecting $10^7$ OH3 cells suspended in matrigel. In summary, there is a moderate but statistically non-significant effect of adding oestradiol. In view of this, SCID mice, matrigel and low dose oestradiol pellets were routinely used in order to ensure conditions for 100% take rate of the parent OH3 cell line. This allowed comparison with microcell hybrids.

**Figure 5.13 Comparison of OH3 cell line subcutaneous xenografts grown in SCID mice in matrigel with or without low-dose oestradiol pellets**

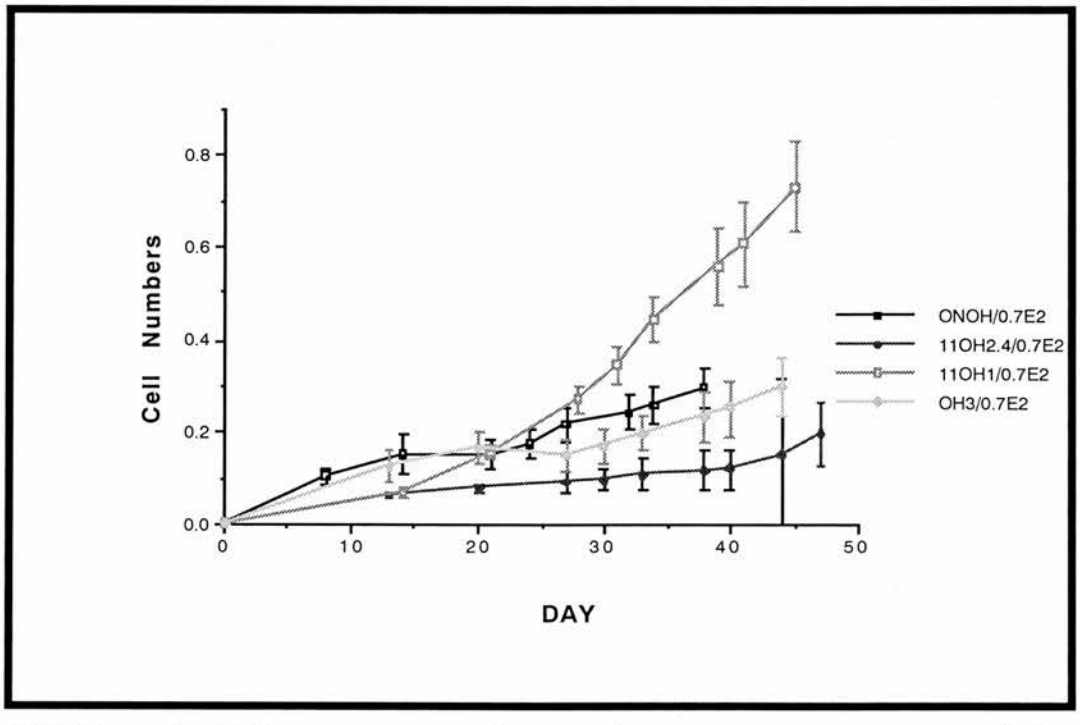



*E2 = 0.72 mg oestradiol pellet used. 4 cell/matrigel injections in each arm*

*5.2.6.1 11OH microcell hybrids*

Having established the optimal conditions for xenograft growth using the parent OH3, clones form the 11OH1 and 11OH2 fusion experiments were directly tested against *neo*-transfected and "neutral" chromosome transfer hybrid clones to observe the effect of chromosome 11 introduction into OH3 (see Figure 5.14). Note that 11OH2.4 was growth delayed but not suppressed for tumorigenicity. Furthermore, the same pattern was initially observed for 11OH1 but then growth accelerated, mirroring the pattern observed in the *in-vitro* growth experiments (see Figure 5.9), providing further evidence for a divergent mixed cell population in 11OH1.

**Figure 5.14 Subcutaneous tumorigenicity of 11OH1 and 11OH2 using low dose oestradiol pellets (0.7 mg) in SCID mice**

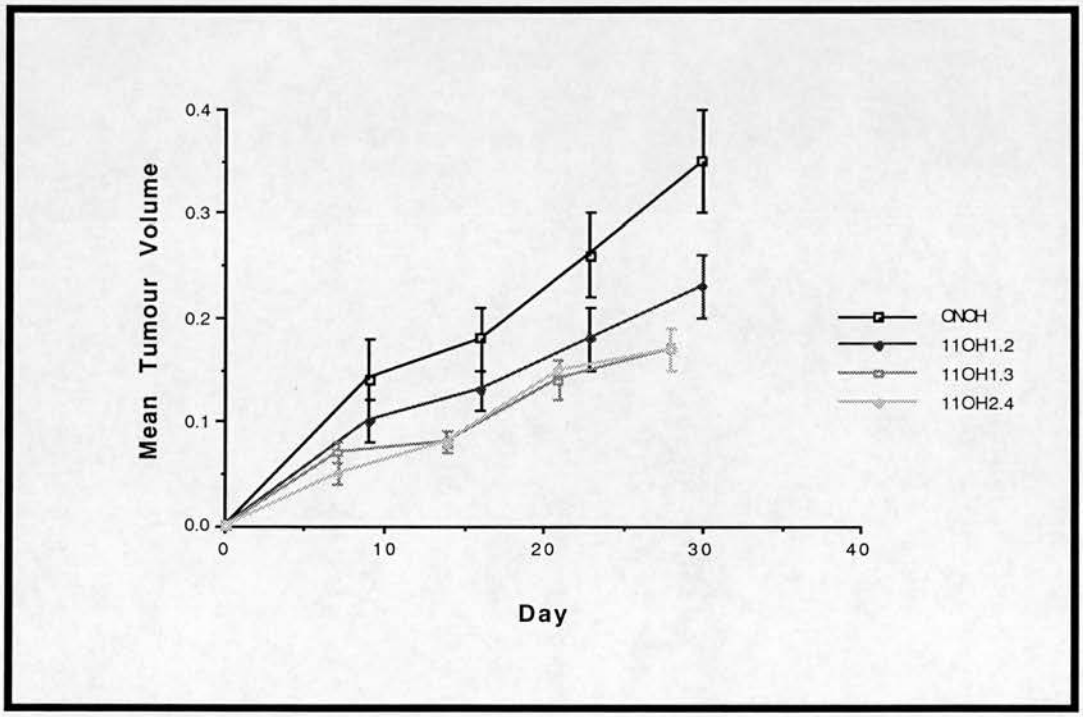



*ONOH and OH3 = controls. Each point represents mean of 10 tumours, error bars represent standard error for each cell line*

Two clonal sub-lines were derived from the 11OH1 cell, one retaining whole chromosome 11 including 11q24, the other with a 5.5 Mb deletion in 11q24 (see chapter 4). These were tested in SCID mice in

direct comparison with the parent control containing the neutral transferred chromosome from OVCAR3 (ONOH). Obvious growth retardation was seen of the xenografts containing whole chromosome 11 (11OH1.3) as compared with the clones containing either a neutral OVCAR3 transferred chromosome or chromosome 11 with a 5.5 Mb deletion in 11q24 (11OH1.2) (see figure 5.15). As can be seen, 11OH1.3, derived from 11OH1 which grew rapidly in the experiment shown in figure 5.14, now grows with identical characteristics as 11OH2.4. This strongly supports the view that we have isolated suppressed and non-suppressed clones from the 11OH1 cell line.

**Figure 5.15: Direct comparison of subcutaneous tumorigenicity of 11OH1.2 and 11OH1.3 in SCID mice**



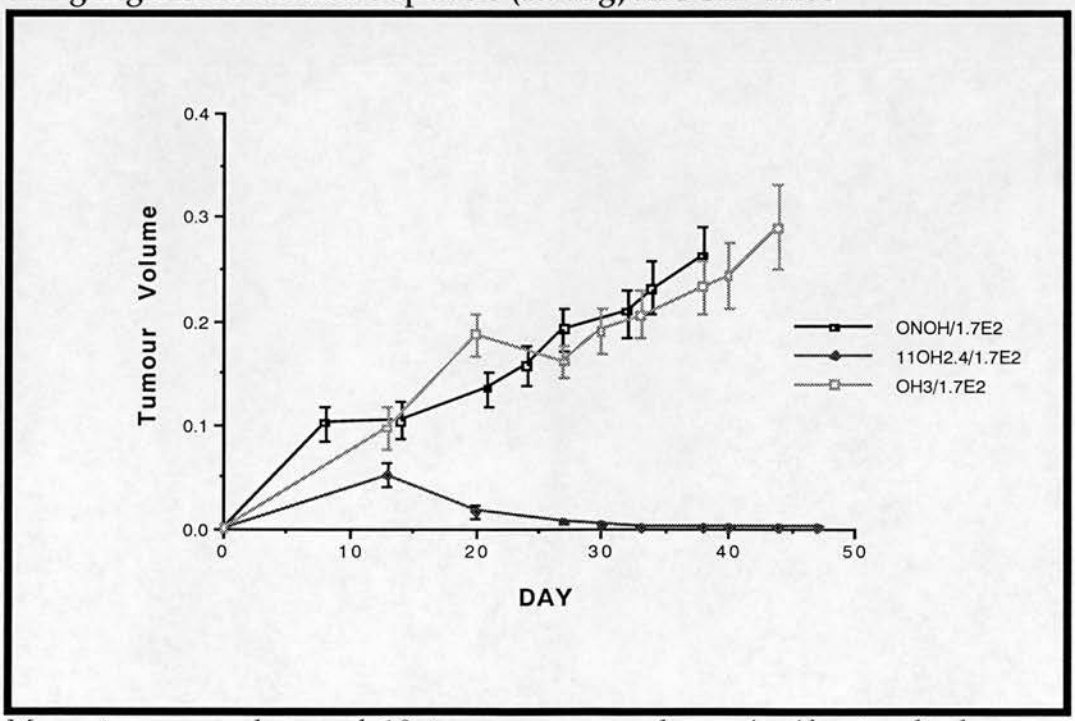

*ONOH = control with neutral transferred chromosome. Each point represents mean of 4 tumours, error bars represent standard error for each cell line*

These findings are consistent with the observation of an ovarian cancer suppressor of growth but not tumorigenicity on chromosome 11, out-with the regions 11p13 and 11q22-qter.

### *5.2.6.2 Effect of Oestradiol*

The effect of high dose oestradiol was directly compared in the same experiment with low dose oestradiol, to look for ways of accentuating differences in the assay. In summary, 11OH2.4 was completely suppressed for tumorigenicity using high dose oestradiol, whereas there was a mildly stimulatory effect of high dose oestradiol on the control cell lines and 11OH1 (figure 5.16)

**Figure 5.16 Subcutaneous tumorigenicity of 11OH2.4 and controls using high dose oestradiol pellets (1.7 mg) in SCID mice**

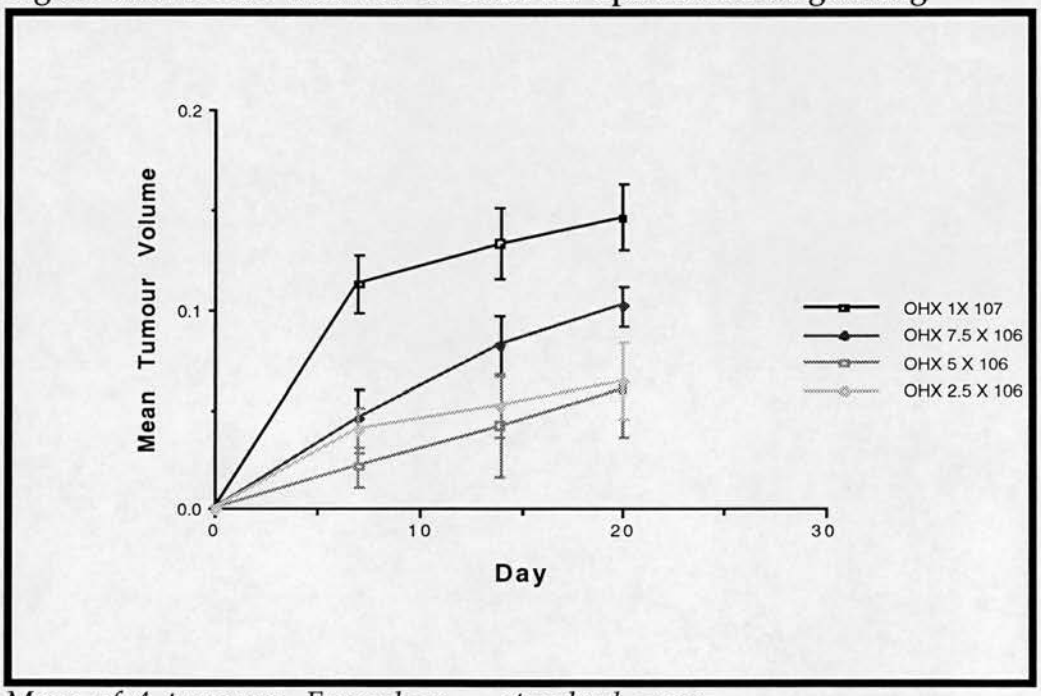



*Mean tumour volume of 10 tumours, error bars signify standard error.*

*5.2.6.3 Xenografts of microcell hybrids derived from the cell line OHX*

Due to the difficulties in establishing convenient xenografting conditions with OH3 (see above), a single xenograft that grew successfully was rescued back and the OHX cell line was established from it. This cell line much more readily formed tumours in SCID mice and down-titration of the cell numbers required for OH3 and derived cell lines for growth are shown in figure 5.17. 5 X $10^6$ were used as the optimal cell number for subsequent xenograft experiments.

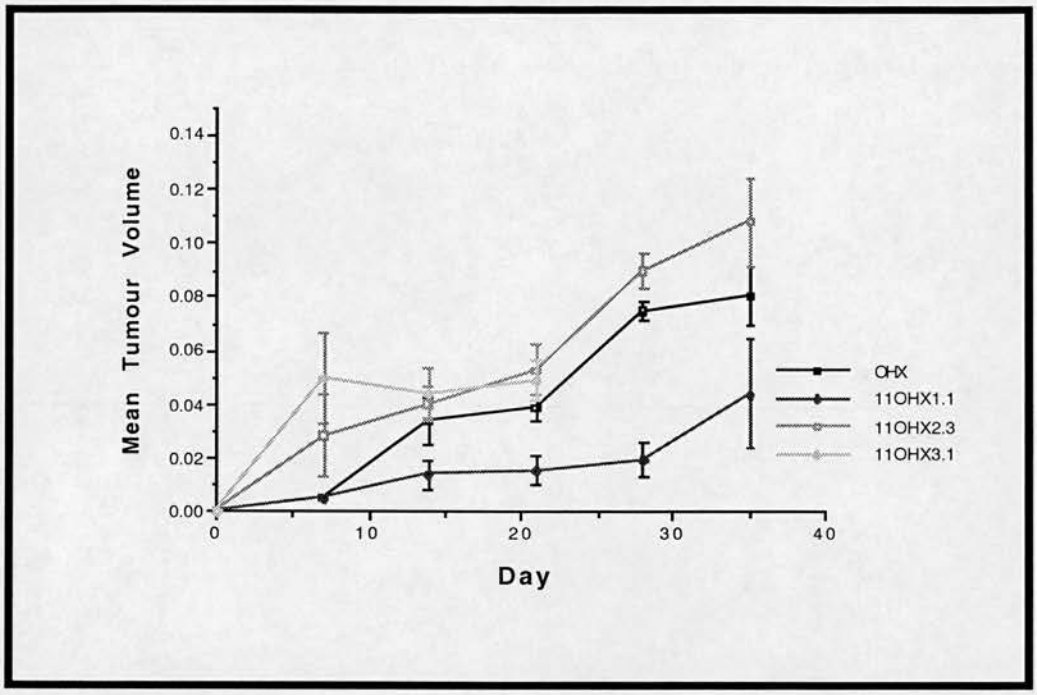
**Figure 5.17: Down-titration of OHX for optimum xenografting**




*Mean of 4 tumours. Error bars = standard error*

In view of this, three further microcell fusions were carried out transferring chromosome 11 to the OHX recipient ovarian cancer cell line. Clones from two fusion experiments received whole chromosome 11 (11OHX1 and 11OHX3) and partial transfer was achieved in a further experiment (11OHX2). Figure 5.18 shows the relative growth of xenografts derived from OHX microcell hybrids.

**Figure 5.18: Subcutaneous tumorigenicity of the 11OHX series of microcell hybrids**




*Mean of 6 tumours. Error bars = standard error*

### 5.2.7 Assays for other components of the cancer phenotype

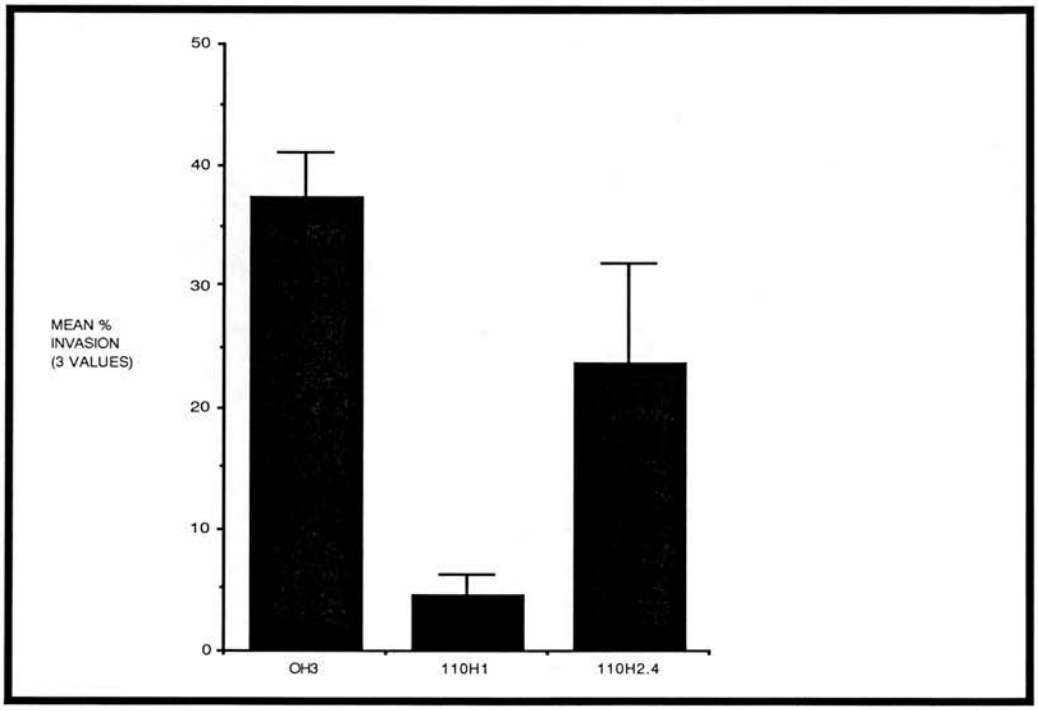
The preceding LOH analysis suggested that advanced stage and poor prognosis were associated with loss of the 11q24-q25 region. Furthermore, LOH analysis differentiated between early and advanced cancer, not benign or malignant tumours. The above tumorigenicity experiments confirmed that growth of the chromosome 11 hybrids was generally delayed rather than suppressed (although it was possible to suppress tumorigenicity of the hybrids selectively under certain conditions). These factors suggested one or several progression-suppressors located on chromosome 11. In view of this, a series of experiments were performed to assess the role of chromosome 11 in suppression of other components of the cancer phenotype; i.e. tumour cell invasiveness/metastasis and components of this pathway such as tumour cell attachment and migration.

### *5.2.7.1 Invasiveness of OH3 and the 11OH microcell hybrids in matrigel*

The initial experiment to assess tumour cell invasion into matrigel produced an unexpected result. In summary, although both 11OH2.4 and 11OH1.1 were suppressed for invasiveness in matrigel, 11OH1.1 was much more suppressed in its ability to invade than 11OH2.4.

This is demonstrated in Figure 5.19

**Figure 5.19 Comparison of matrigel invasion of OH3 (control) with 11OH2.4 and 11OH1**

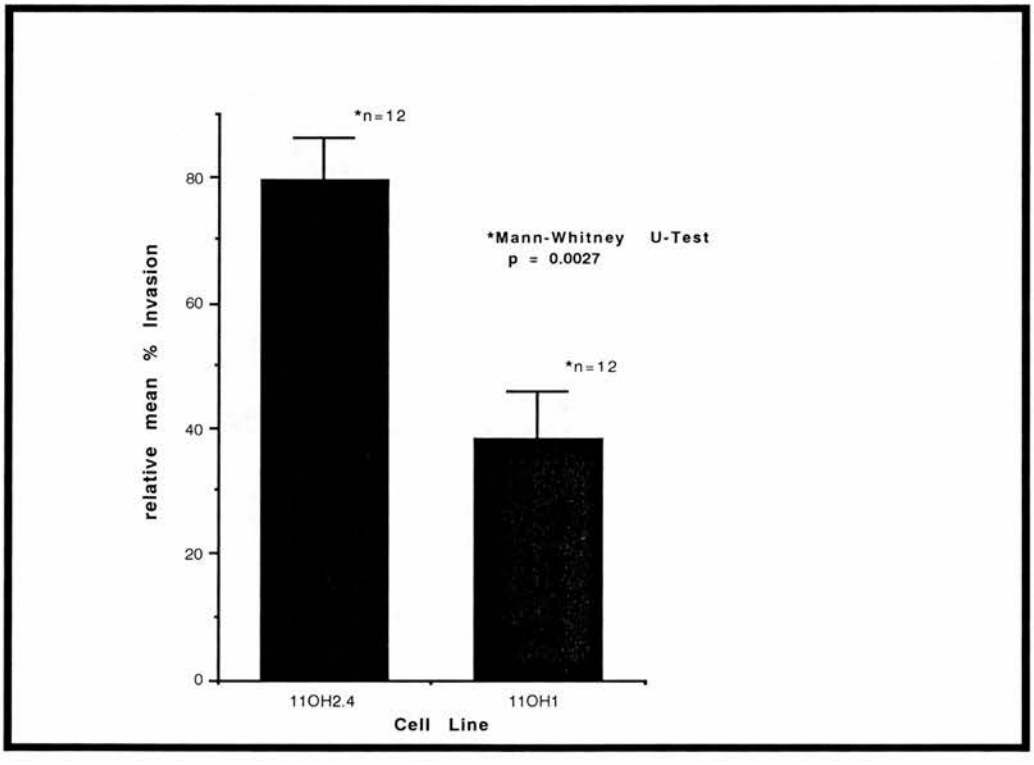



*11OH2.4 (del 11q22-qter) and 11OH1 (whole 11 retained) compared in 48 hour matrigel invasion assay. Error bars represent standard deviation*

In view of this finding, it was imperative to analyse the relationship between 11OH2.4 and 11OH1 in terms of invasiveness by performing more invasion assays. However, small differences in preparation of the matrigel with minor batch to batch variations produced large differences in the actual percentage invasiveness. However, when the

invasiveness 11OH1 was expressed as a percentage of the invasiveness of 11OH2.4, there was remarkably little variation, allowing the pooling of 4 experiments in triplicate directly comparing these two cell lines, and indeed there are precedents in the literature for analysing the data in this way (Tanaka et al, 1996). Within any single experiment, the most invasive sample is given a normalised relative invasiveness value of 100% and the other samples are given a relative invasiveness index as a percentage of this value). Figure 5.20 presents the data from these 4 experiments in triplicate directly comparing 11OH2.4 with 11OH1.

**Figure 5.20 Matrigel invasion assay comparing 11OH1 and 11OH2.4: 12 values from 4 separate experiments**



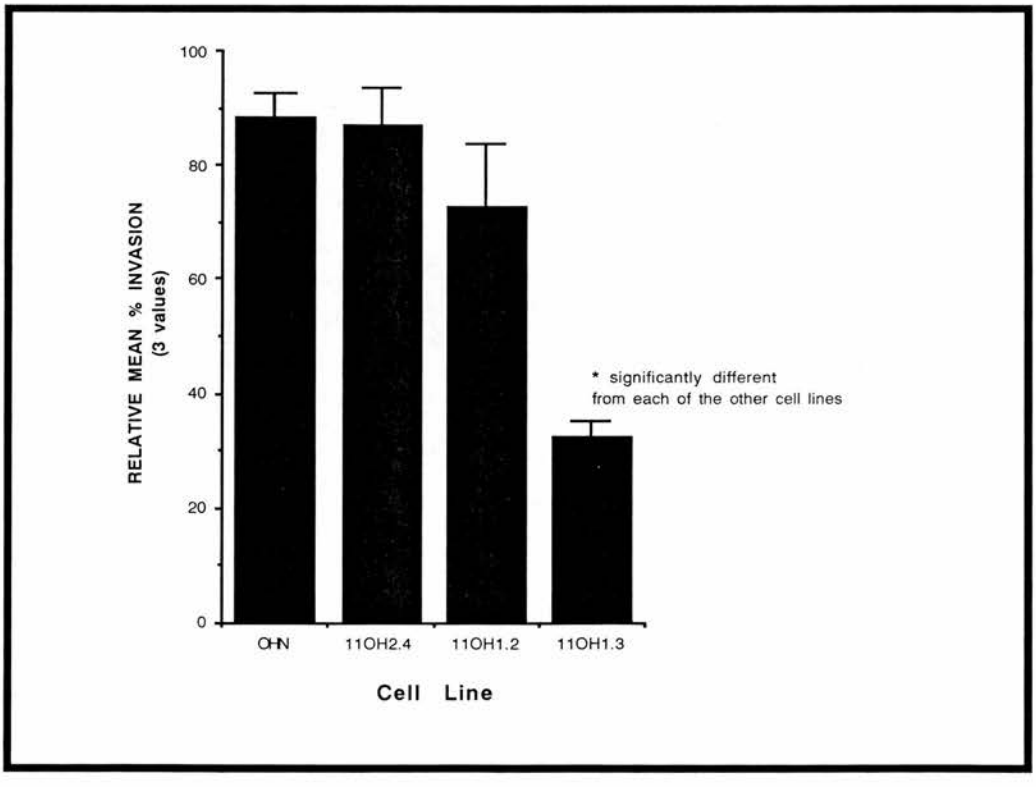

*Error bars represent standard deviation*

As can be seen, a highly statistically significant difference was revealed using the non-parametric Mann-Whitney U test, and this suggests that an invasiveness suppressor function can be mapped to either the 11q22-qter region or the 11p13 region.

In view of the altered growth characteristics observed for 11OH1, two clones were derived as sub-lines, 11OH1.2 and 11OH1.3. These clones showed distinct morphological differences (see Figure 5.7) and differed only in a 5 Mb deletion at 11q24-q24 (overlapping over 4 Mb with the region identified by LOH) and deletion of 11p13. It is possible that the 11p13 region is responsible for the observed morphological differences. However clone 11OHX1.3 has the same morphology as 11OH1.2 yet it retains the 11p13 region and has lost the 11q24 region (see figure 4.16). This suggests that the phenotype of invasiveness inhibition can be ascribed to the 11q24 region.

Direct comparison of the invasiveness of 11OH1.2, 1.3, 2.4 and the parent control is presented in Figure 5.21.

**Figure 5.21 Comparison of matrigel invasion of 11OH1.1/1.2 with control cell line**




*Error bars represent standard deviation*

Importantly, clone 11OH1.3 is significantly inhibited for invasiveness compared with 11OH1.2 (see Table 5.7) differing only in a small deletion at 11q24-q25 overlapping with the region of interest defined by LOH and demonstrating marked morphological differences from 11OH1.3.

**Table 5.7 T-tests comparing invasiveness differences between cell lines from Figure 5.17**

| | **11OH2.4** | **11OH1.2** | **11OH1.3** |
|---|---|---|---|
| **OHN** | p=0.9, NS | p=0.25, NS | p=0.0004 |
| **11OH2.4** | | p=0.31, NS | p=0.0014 |
| **11OH1.2** | | | p=0.024 |

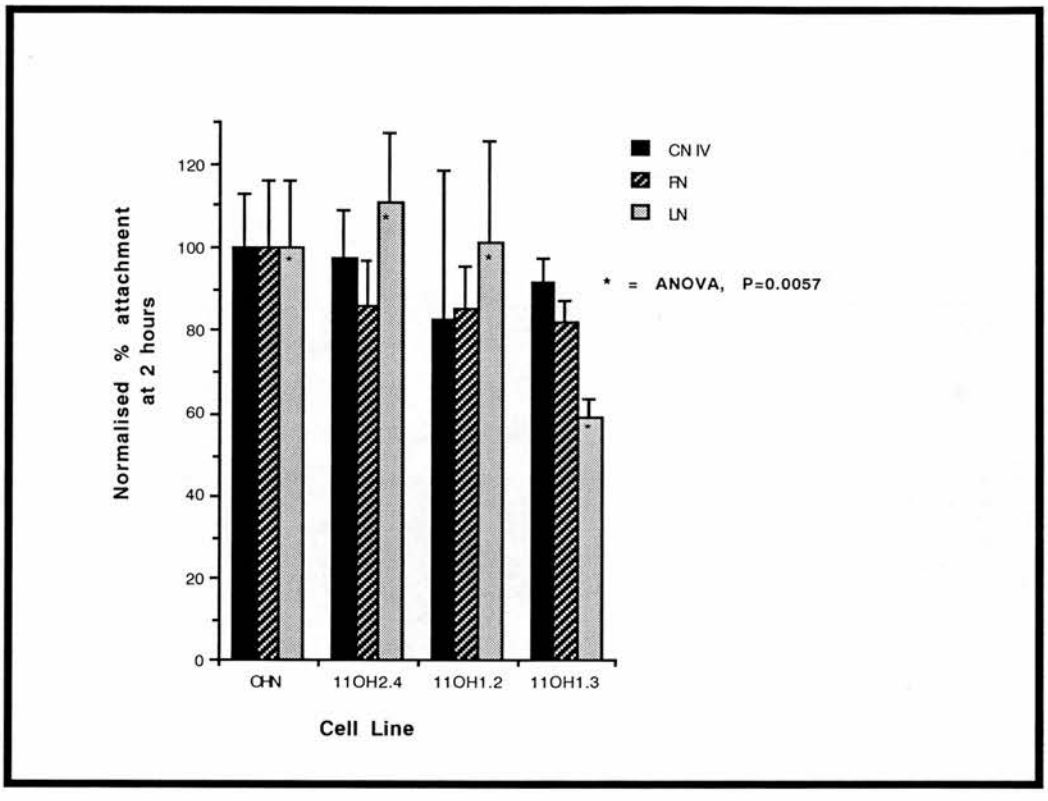
*Two-tailed p-values are given. NS= non-significant.*

In order to further define which components of the invasiveness process are responsible for the observed inhibition effect associated with chromosome 11q24-q25, the effect of individual components of matrigel extra-cellular basement membrane were individually tested in cellular attachment and migration assays, in order to document differences between the hybrid clones and the parent/control cell lines.

### *5.2.7.2 Cellular attachment assay*

The ability of the cell lines to attach to individual components of the extra-cellular matrix was tested by radioactively labelling the cells with chromium and then allowing them to attach to plastic wells coated with the individual matrix components for 2 hours. Figure 5.22 presents this data, and in summary, microcell hybrid 11OH1.3, containing whole chromosome 11 was significantly and specifically inhibited in its attachment to laminin, whereas the other cell lines were not. This specific inhibition of attachment was not seen with collagen IV or fibronectin.

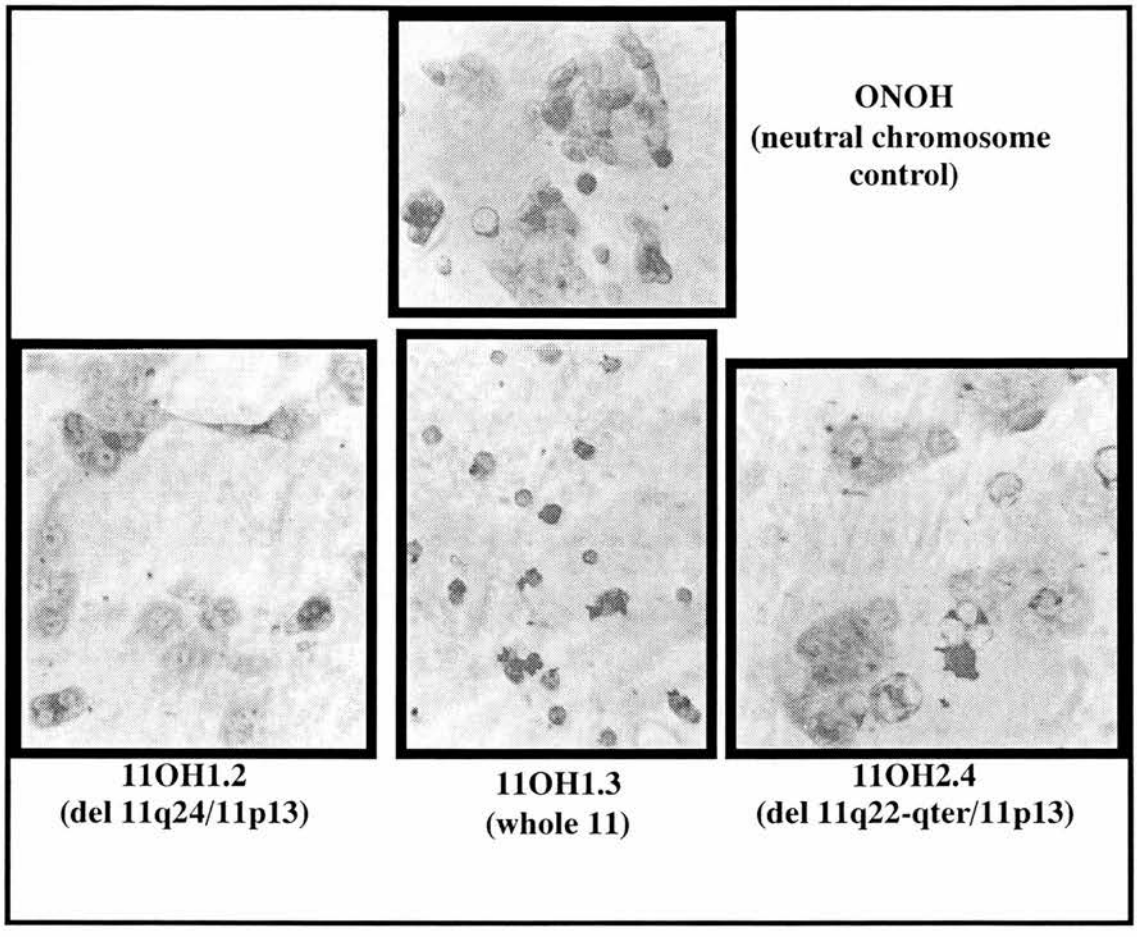
**Figure 5.22 2 hour radioactive chromium attachment assay comparing control with microcell hybrids**




*CN IV=collagen IV, FN=fibronectin, LN=laminin. Attachment values are normalised for control OHN. Hybrid clones are expressed as % attachment in relation to OHN. Error bars signify standard deviation from a quadruplicate derived mean.*

To confirm this observation, plain photomicrographs were taken in two separate further experiments and these confirmed obvious qualitative differences occurring specifically on the laminin coated plates. After 24 hours 11OH1.3 remained rounded up whereas 11OH1.2, 11OH2.4 and the parent cell line ONOH had stuck down and spread out in a strikingly different way (see Figure 5.23).

**Figure 5.23 Morphology after 24 hour attachment of control cell line and microcell hybrids to laminin coated plastic wells**

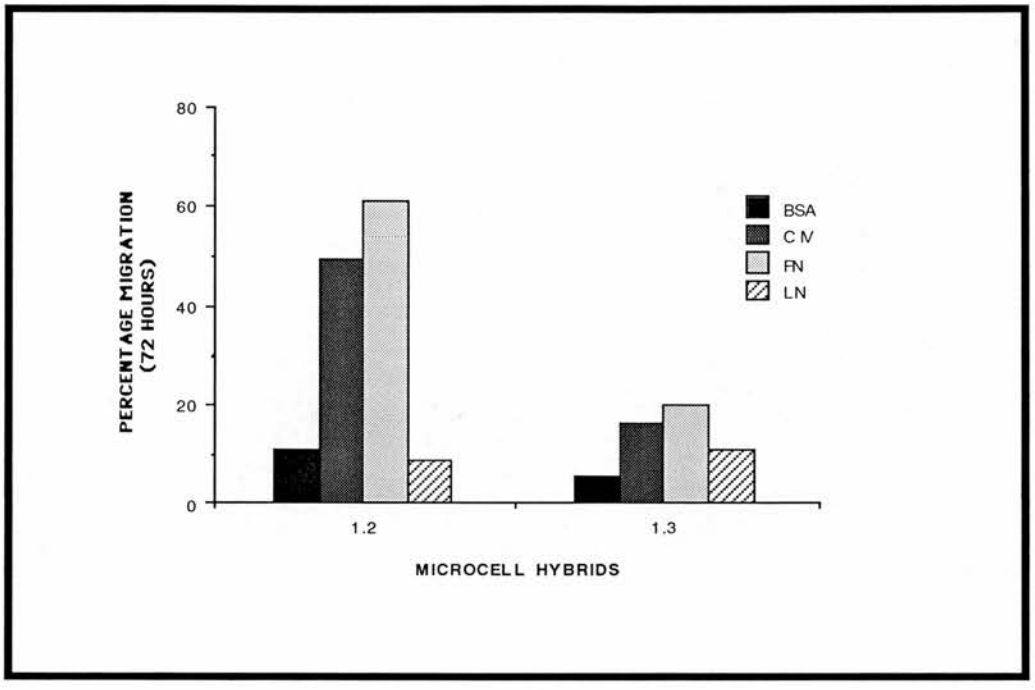



*5.2.7.3 Cell migration assay comparing microcell hybrids*

Using 8 μm pore size Nuclepore filters in transwell chambers whose under-surface had been coated with components of the extracellular matrix, an assessment was made of migration of the cell lines over 72 hours in response to the ECM components. In summary, collagen IV and fibronectin but not laminin generated a haptotactic stimulation of migration over baseline (bovine serum albumin, BSA) in microcell hybrid 11OH1.2. This stimulation of migration was abrogated in hybrid 11OH1.3 (which contains whole transferred chromosome 11)

suggesting that this component of invasiveness could be ascribed to the same region of the chromosome. This data is summarised in Figure 5.24

**Figure 5.24 72 hour transwell migration assay**




*BASS bovine serum albumin (basal migration), LN=laminin, CIVET collagen IV, FN= fibronectin.*

Note that laminin does not stimulate migration in hybrid 11OH1.2, which is in contrast to the promotion of cell adhesion in this hybrid as determined by the attachment assay. This experiment was only performed once due to time constraints and requires repetition.

## 5.3 OVCAR3 chromosome 12 microcell hybrids

Several fusion experiments were performed to transfer chromosome 12 to OH3 (see chapter 4). In two of these, rapid senescence occurred of the clone at 10 weeks. In two other fusions, immortal cell lines were isolated.

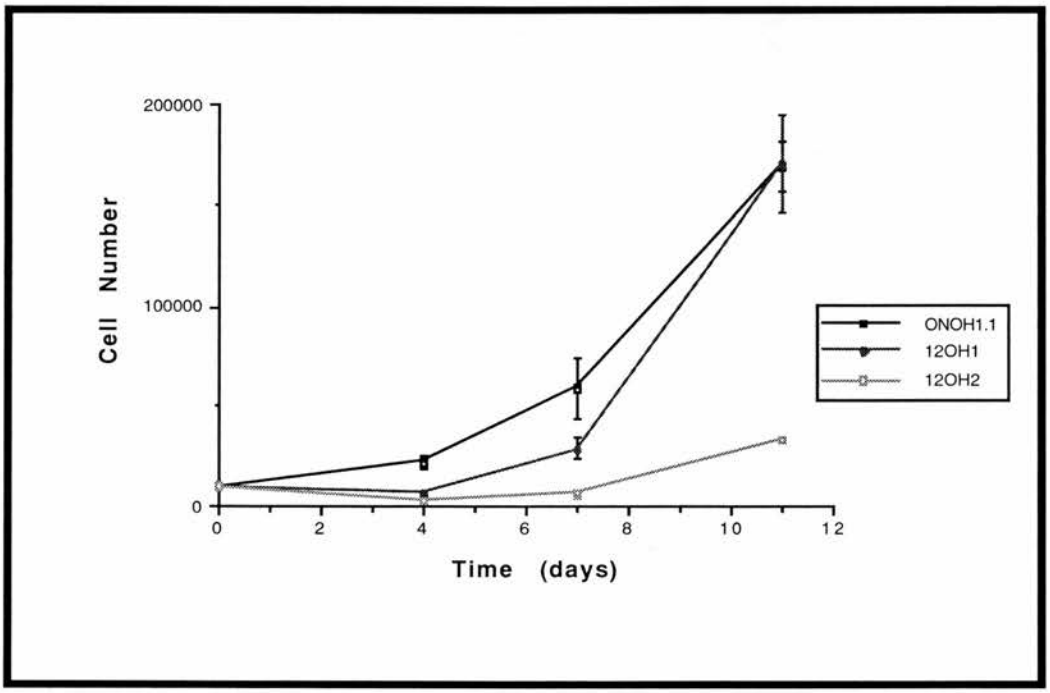
### 5.3.1 Morphology

Morphology of the chromosome 12 hybrids was similar to parent OH3.

### 5.3.2 *In-vitro* growth of microcell hybrids

*In-vitro* growth showed that one hybrid was growth inhibited but the other was not. Figure 5.25 presents this data.

**Figure 5.25 Growth of 12OH series of microcell hybrids**




### 5.3.3 Cell cycle analysis

Flow-cytometric cell-cycle analysis was performed using propidium iodide staining of cells. Table 5.8 demonstrates that no obvious cell-cycle effect was associated with the introduction of chromosome 12 into OH3.

Table 5.8 DNA-FACS analysis of 12OH microcell hybrid series

| | 12OH1 | 12OH2 |
|---|---|---|
| G1 | 57% | 56% |
| S | 22% | 30% |
| G2+M | 21% | 14% |
| PROLIFERATIVE INDEX | 43% | 44% |
| PLOIDY | 1.7 | 1.72 |

### 5.3.4 Apoptosis

No evidence was found for significant induction of apoptosis. Table 5.9 shows FACS-AnnexinV analysis of the 12OH series of microcell hybrids

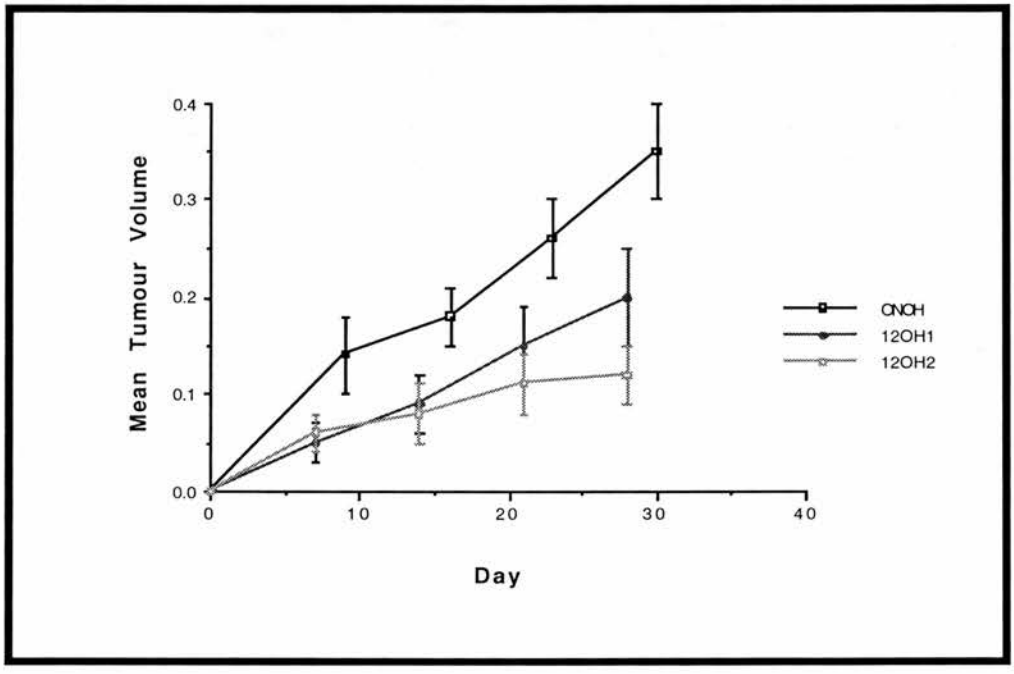
Table 5.9 Annexin V-FACS assay for 12OH series of hybrids

| Cell Line | % Apoptosis (Annexin V +; PI -) |
|---|---|
| Colo 205 | 8% |
| ONOH1 | 5% |
| 12OH1 | 4% |
| 12OH2 | 8% |

### 5.3.5 Xenograft analysis

Microcell hybrids 12OH1 and 12OH2 were xenografted in SCID mice in a direct comparison with ONOH (microcell hybrid with neutral transferred chromosome from OVCAR3). 1 X $10^7$ were injected with matrigel sub-cutaneously into SCID mice. As can be seen from figure 5.26, 12OH2 was not suppressed for tumorigenicity, but tumours were much smaller compared with ONOH. 12OH1 was also growth retarded but to a lesser extent.

**Figure 5.26 Comparison of tumorigenicity of chromosome 12 microcell hybrids with parent control microcell hybrid.**




*mean of 6 tumours. Error bars represent standard error*

# 6. DISCUSSION

## 6.1 Preamble

Ovarian cancer kills women. Its high mortality is related to its late presentation. We know relatively little about how it develops at the molecular level, and much work is still required to provide us with a comprehensive grasp of its molecular pathophysiology. The prospect of fresh conceptual insights from a functional and molecular genetic approach leading to novel therapeutic strategies is a powerful motivating factor for any researcher.

This work is an exploration of the role of chromosome 11 disruption in the development and progression of malignant neoplasia of the human ovarian surface epithelium. Several reports point to the importance of the p arm of chromosome 11 (see "Introduction"). The role of the q arm in ovarian cancer is much less well defined and this thesis focuses on the long arm particularly.

The power of LOH analysis resides in its capacity to sample a representative clinical population for evidence of restricted chromosomal re-arrangement. The ability to identify associations of LOH with adverse clinicopathological features directly relates to the quality control of staging, histopathological and survival data. In this respect, access to blood-tumour pairs from the Edinburgh Combined Gynaecological Oncology clinic brings with it standardised operative technique and staging, and standardised histopathological reporting with minimisation of the inter-observer variability of both. Regular follow-up and standardisation of primary and salvage non-surgical therapy has produced a closely followed cohort of patients in whom one can begin to identify a multitude of heterogeneous phenotypes (morphological, pathophysiological or prognostic) that are involved in the multistep pathway to ovarian carcinogenesis. Univariate analysis can then identify phenotypes (or "factors") which segregate significantly with LOH at particular loci. These LOH associations are by their nature preliminary and mark only the beginning of serious investigation.

Unlike the use of polymorphic microsatellites in linkage analysis of constitutive disorders, a positional cloning approach based only on loss of heterozygosity analysis (as opposed to homozygous deletions) utilising malignant neoplasms is a risky approach since there is a high chance that LOH may simply be due to non-specific genome instability associated with disruption of a few genes controlling generalised genomic stability. The need to gather evidence that these LOH associations house biologically active suppressor functions with a role in the development and progression of clinical cancer is therefore of importance. It follows that functional approaches are necessary to make an assessment of the relevance of these regions. We can expect that this will not only increase confidence for but also narrow down the region of interest. Furthermore, it raises the possibility of a direct functional cloning approach (see later).

In order to unambiguously assign function to regions of chromosome 11, the principles of somatic cell genetic analysis have been employed in the technique of microcell mediated chromosome transfer, whereby chromosome 11 as a whole or as a spontaneously fragmented part was transferred to an ovarian cancer cell line. The transferred fragments were mapped and an attempt was made to assign function to intervals of the chromosome. This section will discuss the evidence for important LOH and functional findings that bring together these independent strategies in a convergent and complementary way to highlight the importance of a small region in the distal part of chromosome 11q involved in the progression of ovarian cancer.

**Loss of Heterozygosity and its associations**

With a panel of 10 highly informative, well distributed, accurately mapped polymorphic microsatellites (MSPs), significant levels of chromosome 11 LOH were detected in a well characterised population of 60 patients with ovarian tumours, many of whom had been previously studied with respect to their chromosome 17 status by Dr Diana Eccles, my predecessor (Eccles et al, 1990).

Although the term LOH is used interchangeably with allele imbalance in this work, it must be borne in mind that imbalance can also be a consequence of allele specific amplification and need not necessarily imply deletion of a region of DNA. Furthermore, amplification of a region of DNA is not mutually exclusive with loss of function at a tumour suppressor locus; loss of a chromosome or subchromosomal region may occur with reduplication of the other allele/chromosome, and amplification of a region of DNA is not necessarily associated with gain of function if accompanying inactivating mutations are involved.
In contrast to findings with chromosome 17 (Steel et al., 1994; Foulkes et al, 1993; Tavassoli et al, 1993), where whole homologue or whole arm loss is common, interstitial and small terminal deletions are in fact more common in chromosome 11 in this same group of ovarian tumours. A highly significant association between allele imbalance on 17p and 17q in this material was noted (see results). No such association is observed for imbalance on 11p and 11q as a whole, and this argues for caution in the general interpretation of allelotyping data that utilise only one or two loci per chromosome arm where no previous biological hypothesis associates that chromosome arm with involvement in neoplasia.
However, significant relationships for LOH between distant chromosome 11 loci were observed; not only between adjacent sites (which are likely to reflect larger deletions) but also between distant loci despite retention of intervening loci. These pairs of loci may harbour genes which are co-operatively inactivated as part of a multistep process.

## 6.2 Loss of Heterozygosity

### 6.2.1 Allele loss of the short arm of chromosome 11 in ovarian cancer

In contrast to previous reports (Kiechleschwarz et al., 1993; Zheng et al., 1991), I found no significant association between differentiation grade and LOH at the 11p15 region (despite a rate of 67% LOH) in our sample of EOCs, and this may reflect the lack of uniformity in ovarian

cancer grading methodology. I could not confirm the previously described association between 11p15 LOH and advanced stage disease (Viel et al., 1992), although there appeared to be non-significant trends for both these parameters. Significant LOH rates at 11p13 near the site of WT1 have been detected in several studies, although direct analysis of the WT1 locus suggests that it is not the gene involved (Bruening et al., 1993; Viel et al, 1994). There was also evidence of significant LOH at D11S922 in benign and borderline tumours. With this high level of loss in EOCs, and without significant correlations with advanced disease/poor prognosis subgroups, the likelihood is that an estimated 18.6 Mb interval within 11p15 houses a gene involved at an early stage in ovarian carcinogenesis, occurring as part of the development of benign and borderline tumours and also detectable at roughly similar rates in adenocarcinoma. Allele loss at D11S569 (11p15.3) is low (12.5%) in benign and borderline tumours, but is much higher in carcinomas, and there is no difference in LOH rate between early and advanced FIGO stage adenocarcinomas. This raises the possibility of a second locus at 11p15.3 which is inactivated as part of the development of frank adenocarcinoma (albeit at an early stage of adenocarcinoma development).

### *6.2.2* The centromeric parts of chromosome 11

The 11p12-q14.3 region (which is a large region containing the centromeric half of 11q), although exhibiting high levels of loss, does not appear to segregate significantly with any particular parameter, although there is a non-significant trend towards LOH in association with better prognosis tumours was associated with favourable clinicopathological parameters. Higher LOH rates are observed in those with mucinous histology, early FIGO stage and well/moderately differentiated tumours. Higher rates of allele loss at D11S873 are also seen in those patients remaining. These findings suggest that some well differentiated, early FIGO stage carcinomas may belong to a

genetically distinct sub-category of EOC rather than being simply precursors of aggressive late stage disease, and that allele loss in the 11p12-q22 region may confer changes incompatible with rapid progression of the disease, e.g. deletion of an oncogene locus essential for tumour progression. It is possible that LOH detected in this region could reflect amplification of the 11q13 region similar to that observed in breast cancer (Karlseder et al., 1994) and this possibility has not been ruled out, although previous studies of this region in ovarian cancer (Foulkes et al., 1993) suggest that amplification occurs infrequently. However, the explanation for our findings of better prognosis associated with allele loss at this locus remain obscure and require further work.

### 6.2.3 Proximal 11q LOH and the progesterone receptor locus

The absence of LOH at D11S873 and D11S35 specifically in endometrioid adenocarcinoma is of considerable interest, though the finding approached statistical significance only for the latter MSP ($p=0.04$).

D11S35 lies about 160 Kb from the site of the progesterone receptor gene (PGR). At least 6 previous studies have reported that endometrioid tumours contain progesterone receptor (PR) levels that are elevated relative to other histological types (Slotman and Rao 1988). Furthermore, there is evidence that in breast cancer, gene dosage plays a secondary but significant role to regulatory change for hormone receptor levels; tumours that were cytogenetically 6q-/11q- had half the average ER or PR value of tumours without losses on 6q or 11q (Magdelenat et al., 1994).

Further analysis of the Edinburgh patient cohort demonstrated that allele loss close to the progesterone receptor gene on chromosome 11q22 was associated with low tumoral PR content suggesting the possibility that genomic structural disruption including or flanking

the progesterone receptor gene may have a significant role in the dysregulation of PR content of at least some ovarian cancers.

That allele loss at the PR gene locus may genuinely disrupt PR expression is also suggested by the observation that when tumours with LOH at this locus are excluded from the informative sample, a significant correlation between tumoral ER and PR content is revealed, indicating that in many of these heterozygous tumours, the regulatory link between oestradiol, ER and PR is intact, and that the converse is true in those with LOH at the PR gene locus. Although trends also are observed for distantly physically linked (D11S935; 11p) and unlinked (NM23; 17q) markers, they do not reach conventional significance levels nor are the trends substantially different from each other. The fact that there are non-significant trends is consistent with the likelihood that PR gene disruption will not be the only mechanism of tumoral PR down-regulation.

Not all previous literature is supportive of these observations. Fuqua et al (1991) investigated the status of RFLPs within the PR gene in a large series of breast tumours and found no correlation with PR expression. Additionally 0/5 informative breast cancer normal/tumour pairs exhibited allele loss using one of these RFLPs, and the authors concluded that PR gene rearrangements were not a significant mechanism for the alteration of tumoral PR content. However, the extent of contaminating normal stroma (containing constitutive DNA) in these tumours was not discussed, and the 6 human breast cancer cell lines tested in the study were all homozygous at the reported RFLPs. However, more recently, it has been shown that alterations in methylation in regions flanking the ER and PR receptor genes is associated with reduced expression of these genes in breast cancer ( Lapidus et al, 1996).

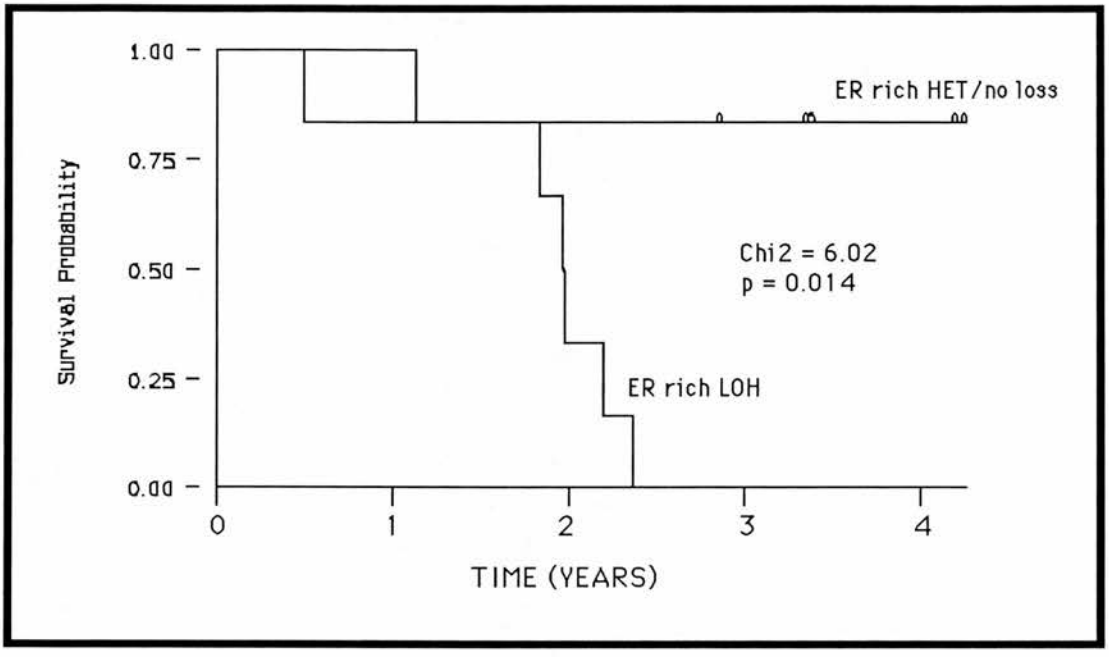
The observed tight link between D11S35 LOH and loss of PR expression may be due to variations on the classical Knudson two-hit model (Knudson, 1971).

Clearly, analysis of the PR gene locus and RNA species in these tumours is required to demonstrate the nature of these abnormalities, since the Kd68 anti-PR antibody may not detect truncated forms of the PR protein.

Analogous work with the oestrogen receptor in breast cancer has demonstrated an ER variant lacking exon 5 of the hormone-binding domain in ER-negative/PR-positive breast cancers that constitutively activates the oestrogen response element (Fuqua et al, 1991b). A truncated ER species which inhibits the binding of ER to its response element has been identified in ER-positive/PR-negative breast tumours (Fuqua et al, 1992), and an ER variant co-species lacking exon 3 of the DNA-binding domain was identified from ER-positive tumours which was unable to function as a transcriptional inducer (Fuqua et al, 1993).

Kaplan-Meier survival analysis in the small subgroups of our series must be treated with caution given the sample sizes. Nevertheless, it is interesting that patients with ER-rich tumours and allele loss close to the PR gene had particularly poor survival, all 6 patients having died within two and a half years; whereas those with ER-rich tumours that retain heterozygosity at the PR gene locus have particularly good survival with 5 of the 6 patients alive beyond three years (figure 6.1).

**Figure 6.1 Survival of ER rich patients according to D11S35 LOH status**




A prospective analysis in a larger series of patients is warranted to confirm this potentially important prognostic marker.

Our finding that none of the endometrioid tumours had loss of heterozygosity close to the PR gene is intriguing, and consistent with the many reports that this histological subtype is associated with higher tumoral PR content (Slotman and Rao, 1988) and the finding in one study that endometrioid tumours can have a 54% response rate to progestin therapy (Rendina et al, 1982)

The analysis of PR content in relation to survival may well be complicated by the presence of LOH in association with abnormal PR protein which is undetected by antibodies. Removing tumours with LOH from our series, and examining those that had retained heterozygosity resulted in the survival patterns that would have been expected; patients with ER-rich and PR-rich tumours exhibited a survival advantage

In conclusion, structural alteration at the PR locus on chromosome 11q22 may represent a significant mechanism for the dysregulation of

tumoral PR content. Subsequent to publication of this work (Gabra et al, 1995b), further analysis of PR gene structure, expression, and associated clinical correlations in these tumours to determine the mechanism(s) involved are currently underway in our laboratory.

### 6.2.4 Definition of a distal locus at 11q24 associated with poor prognosis

As part of the initial study, the finding of extensive allele loss distal to 11q23.3 (Foulkes et al., 1993) was confirmed and extended (both in numbers and chromosomal position), with 67% of EOCs exhibiting LOH at 11q23.3-q24.1 in our sample. The proximal marker (D11S925) in this region mapped about 1.2 Mb telomeric to the most distal marker in Foulkes' study. Loss of heterozygosity at the distal MSP (D11S912) at 11q24.1 was significantly associated with adverse survival and advanced stage, although the latter p value at 0.035 was borderline.

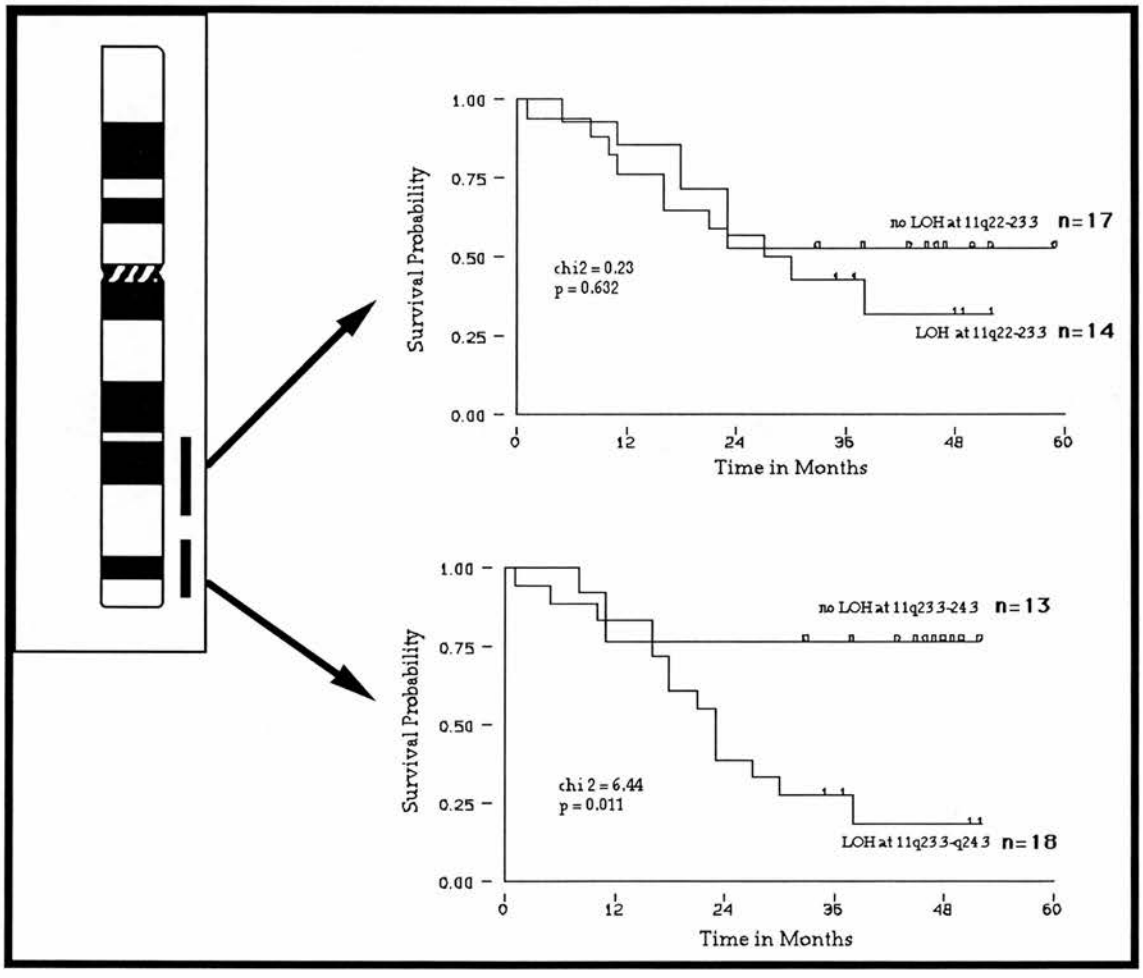
No borderline tumours exhibited allele imbalance at D11S912. These initial findings suggested that a TSG primarily acting as a "progression suppressor" might be located at 11q23.3-24.1(or telomeric), and that its inactivation could be a significant late event in the pathogenesis of epithelial ovarian cancer. The publication of this work (Gabra et al, 1995a) which confirmed and extended Foulkes' analysis prompted a more thorough investigation of this region which determined that there were two loci with high rates of LOH in the distal portion of 11q.

The proximal SRO (which is a large region defined by only two microsatellites in our study) contains the ataxia-telangiectasia and progesterone receptor genes and is lost frequently (in 40% of cases), but does not crudely correlate with survival or other adverse prognostic features in EOCs, although it is associated with low tumoral progesterone receptor content (see above). This proximal region broadly corresponds to a region that has recently been identified in breast cancer (Carter et al, 1994; Hampton et al, 1994; Negrini et al, 1995)

A more telomeric 11q23.3-q24.3 region, excludes the ATM locus and the region recently mapped in breast cancer. The SRO has been

narrowed by this analysis to 8.5 Mb lying between D11S934 and D11S1320 at 11q23.3-24.3 based on recently published physical (Van Heyningen and Little, 1995; James et al, 1994). Kaplan-Meier survival analysis revealed that LOH involving this 8.5 Mb region is associated with poor survival in EOCs (Figure 6.2).

**Figure 6.2 Telomeric 11q loss of heterozygosity and patient survival**




*Regions of chr.11 telomeric LOH are shown in thick vertical lines*

However, when only the advanced stage tumours were considered, the Log rank test did not reach significance, although Kaplan-Meier analysis showed a trend towards worse survival in those with LOH of the distal region. This suggests that the late acting progression-suppressor mentioned above may be located within this telomeric interval.

Interestingly, a paper by Winqvist and colleagues (Winqvist et al, 1995) demonstrated that LOH of telomeric 11q was associated with adverse survival in women with node positive breast cancer. LOH within this region should therefore be subjected to a larger prospective analysis to assess its role as an independent prognostic factor, and its value in assessing survival prospectively. The distal deletion interval currently contains 5 candidate genes: (from centromeric to telomeric) SRPR, ETS-1, FLI1, ZNF1 and NFRKB (James et al, 1994).

The possibility that high frequency non-specific genome instability might be responsible for the observed regional losses was controlled by selecting tumours with chromosome 11 loss preferentially, making it unlikely that the observed survival association in this population could be attributed to random high frequency genetic alteration. Additionally, the retention of heterozygosity telomeric to the 11q23.3-q24.2 LOH region suggested that this distal unit is not simply due to high frequency non-specific telomeric breaks. Analysis of a panel of 14 ovarian cancer cell lines using 6 MSPs from the critical region showed no homozygous deletions. This study was published in early 1996 (Gabra et al, 1996a).

Within two weeks of the publication of this work, the same journal published another paper which had simultaneously and completely independently confirmed these findings using a comparable analysis. Davis and colleagues (Davis et al, 1996) working in Southampton, confirmed my initial study (Gabra et al, 1995a) and defined almost an identical telomeric locus of LOH, overlapping over a region of about 4.5 Mb at 11q24. Although they could not confirm the relationship with advanced stage in their study, they did suggest that it was possible that many of their early stage tumours might in fact have been advanced tumours that were not sufficiently well staged. Finally, the relationship of telomeric 11q LOH and adverse survival in ovarian cancer has now been confirmed by another worker (Georgia Chenevix-Trench, personal communication).

### 6.2.5 Associations of LOH on chromosomes 11 and 17 with clinicopathological features of ovarian cancer.

Extensive chromosome 17 microsatellite and RFLP analysis of this same cohort of patients had been performed (Eccles et al, 1992a; Steel et al, 1993) prior to the chromosome 11 analysis. The data were combined to provide an overview of the role of these two chromosomes in ovarian cancer.

Chromosome 17 and 11 LOH affecting individual polymorphic loci and whole chromosome arms showed significant concordant events and associations with prognostically important clinicopathological features of ovarian cancer. The data presented in this study do not allow interpretation of the prognostic independence of these markers, as only associations can be reported with this type of univariate analysis.

Most striking among the "whole arm" co-loss events was the association between 17p and 17q LOH. This has been described previously in allelotype analyses using fewer polymorphic markers than this study (Foulkes et al, 1993; Osborne et al, 1994) and our findings are also highly supportive of the hypothesis that chromosome 17 whole homologue loss is a very common event in ovarian cancer. In contrast, there was no association between 11p and 11q LOH in the same patient population, demonstrating that although LOH on chromosome 11 is a frequent event, it is unlikely to be explained by whole homologue loss (by mechanisms such as non-disjunction postulated to be important in chromosome 17). There is also an association between telomeric 11q LOH and 17q LOH, which has also been observed for breast cancer (Gudmundsson et al, 1995). The significance of this observation is not clear, although both these regions contain polymorphic markers where LOH is associated with adverse survival.

Significant associations of LOH between individual polymorphic markers were also noted in this study, and for some of these, plausible candidates or biological hypotheses exist (see chapter 4).

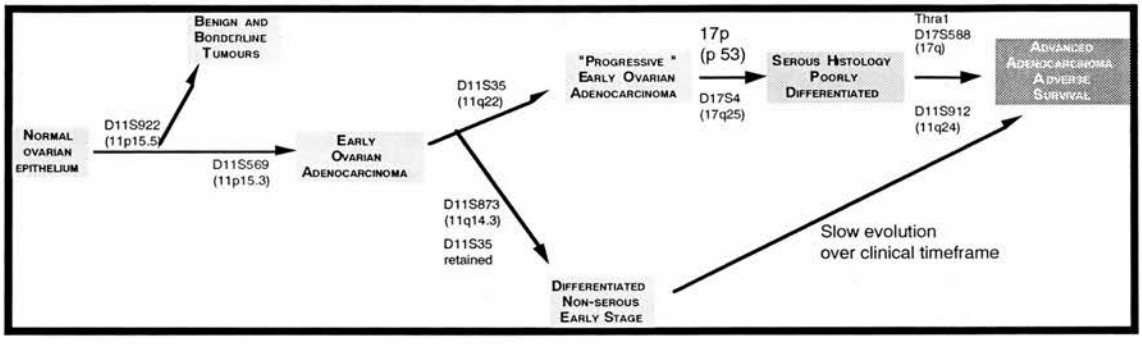
Several observations were made in this analysis with regard to the association of LOH with prognostically important clinicopathological factors in ovarian cancer. Whole arm loss was significantly associated with clinicopathological factors only for chromosome 17p. This is perhaps not surprising when one considers that of the four chromosome arms in this study, only 17p appeared to be lost uniformly as a whole arm, i.e. all 17p polymorphic markers underwent LOH together. This may be a reflection of the importance of the p53 gene and its role in neoplasia. The most significant association noted was of 17p LOH with serous ovarian adenocarcinoma. The observation of p53 mutation in serous ovarian cancer is well described in several previous studies (Eccles et al, 1992a; Niwa et al, 1994; Kupryjanczyk et al, 1993; Klemi et al, 1994) 17p LOH is also associated with poor differentiation of the primary tumour and advanced stage at presentation, although these associations are at borderline significance. However, there was no association of 17p LOH with adverse survival. It is of interest that several extensive studies have reported no relationship between p53 abnormality and survival in ovarian cancer (Niwa et al, 1994; Sheridan et al, 1994; Kohler et al, 1993). The only other notable "whole arm" observation was an association of borderline significance between 11q whole arm loss and adverse survival.

An analysis of LOH at individual polymorphic loci with prognostic clinicopathological features revealed several significant associations.

One can speculate that polymorphic markers undergoing frequent LOH, where no significant difference is noted between favourable and adverse clinicopathological features, may represent early events in ovarian carcinogenesis. Conversely, polymorphic markers segregating with adverse clinicopathological phenotypes may be intermediate or late acting events (depending on phenotype). One can begin to construct a preliminary picture of the possible involvement of chromosomes 17 and 11 in a multistep model of ovarian neoplasia

similar that constructed by Vogelstein for colorectal cancer. The model published with this analysis (Gabra et al, 1996b) demonstrates not only multistep but also multipath components in the pathway to ovarian cancer (Figure 6.3).

**Figure 6.3 Integrated model of ovarian carcinogenesis with respect to allele loss of chromosomes 11 and 17.**




In this model, 17p (p53) LOH represents an intermediate or early event; 17q and telomeric 11q LOH represent late events. Although the evidence is inconclusive, I have included D11S35 LOH as an early event in neoplasia, perhaps involving the progesterone receptor, and D11S873 LOH as an early event in generating a genetically distinct, prognostically advantageous, subgroup which presents as the relatively rare early stage form of ovarian adenocarcinoma.

It is clear that this univariate analysis is a crude tool with limits to the extent of inferences that one can draw about ovarian neoplasia. However, it does allow some general observations to be made regarding comparisons of subchromosomal abnormalities in a small but well-defined cohort of patients with ovarian cancer. It is to be hoped that refinement of these regions will provide further mechanistic answers to the observed associations as positional cloning efforts ultimately yield genes involved in these processes.

Considering the LOH studies discussed above as a whole, they have extended the previous observations of distinctive patterns of aneusomy or molecular aberrations in ovarian cancers belonging to different clinico-pathological subgroups. The observations do not imply that LOH at each of the defined regions of chromosome 11 represent independent prognostic factors, although 11q sub-telomere imbalance should perhaps be subjected to a large prospective study.

## 6.3 Microcell mediated chromosome transfer

### 6.3.1 Structural analysis

Having used a clinical resource to define frequent LOH rates at specific loci on chromosome 11 and their association with the clinicopathological features of ovarian cancer, I set out to determine whether or not tumour suppressor genes of importance in ovarian cancer actually reside on chromosome 11.

A normal human chromosome 11 was transferred by microcell mediated chromosome transfer to the ovarian cancer cell line OVCAR3.

OVCAR3 was established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary after combination chemotherapy with cyclophosphamide, Adriamycin, and cisplatin and represents a poorly differentiated ovarian cancer (Hamilton et al, 1983). *In-situ* hybridisation revealed that 5 centromeric spots are observed by chromosome 11 specific alphoid PRINS. Chromosome painting showed that chromosome 11 was markedly rearranged. Flow cytometric analysis and karyotyping showed that OVCAR3 was hypo-tetraploid with a DNA content of 1.8N relative to normal diploid blood lymphocytes. However, microsatellite analysis of OVCAR3 demonstrated homozygosity of all markers suggesting that whole homologue loss of this chromosome had occurred with reduplication. No homozygous deletions in OVCAR3 were detected with the markers used in this analysis.

The normal donor human chromosome 11, tagged with a *neo*-marker was available from two different monochromosome somatic cell hybrids: J1 Cl4 and 556.1.5. J1 Cl4, a CHO cell line proved to be a poor microcell donor under a wide range of conditions. 556.1.5 proved to be a good chromosome 11 donor, with broad tolerance conditions for micronucleation. A *neo*-retrovirus was used to tag this chromosome (Lugo et al 1987), and the retroviral site of integration was 11q14-q22 (Bernard Weissman, personal communication). It was therefore expected that chromosomal retention would cluster around this region, which indeed turns out to be the case (see figure 4.14) with individual microcell hybrid clones variously containing donated chr 11 with fragmentations of the p arm and 11q22-qter but no instances of deletion of proximal 11q.

The potential problem of heterogeneity within the OVCAR3 population might have resulted in microcell hybrid clones (MHCs) with artefactual functional differences from the parent control due to simple clonal selection. In order to address this, a clonal sub-line of OVCAR3 (OH3) was generated by *hyg*-tagging of OVCAR3 and selection of colonies. This clonal sub-line was used as a recipient for MMCT. In order to avoid the possibility that non-specific functional effects were produced by the introduction of the *neo*-marker itself rather than the chromosome 11 into which it was integrated, control cell lines were generated that were dually transfected with *hyg* and *neo*. Furthermore in order to avoid the possibility that the non-specific introduction of chromatin into the cell line produced suppressive effects, a "control" chromosome was transferred to OH3. Initially, chromosome 1 was transferred; however this universally produced extreme growth arrest/ senescence. Chromosome 12 was then transferred and it also produced cell death at about 8 weeks of the entire population of cells. However some clones did avoid death and went on to be developed into cell lines, one of which was functionally suppressed for growth and delayed for tumorigenicity. In view of both

of these chromosomes being functionally active as suppressors for OH3, the ultimate non-suppressing chromosome was transferred: OVCAR3 was transfected with *neo*-plasmid and a clonally derived cell line (ON3) was used as a microcell donor to transfer a *neo*-tagged chromosome to OH3. Since functionally active TSGs are already dysfunctional in OVCAR3, any chromosome transferred from OVCAR3 (ON3) to itself (OH3) should by definition not contain a TSG. These highly appropriate controls behaved as they were expected to, conferring no functional alterations.

Transfer of chromosome 11 to OH3 was confirmed by *neo* PCR and *in-situ* hybridisation. MMCT using J1 Cl4 resulted in a "bystander survival" effect whereby in hygromycin/geneticin dual selective media, the OH3 and J1 Cl4 population protected each other from cell death until the concentrations in the media were increased. When these mixed population clones were expanded and DNA was extracted, both donor and recipient alleles were detected resulting in the erroneous conclusion that chromosome transfer had been successfully achieved. Painstaking analysis was required to prove that these were mixtures of donor contaminant cells with parent recipients. This leads to the view, which I hold strongly, that *in-situ* hybridisation is the best formal proof of chromosome transfer. This has implications for the interpretation of the majority of published functional analyses. Another iconoclastic notion derived from this work was the extent to which transfer of murine sequences occurred in microcell mediated chromosome transfer. In general, this issue is not addressed in most publications concerned with MMCT, or worse, assumed to be irrelevant. The need to identify the incidence of this phenomenon arose since it was implicit that the MHCs would eventually be used to as a difference cloning resource. The question therefore arose of the amount of murine expressing chromatin that could interfere in such difference cloning experiments. By murine cot-1 *in-situ* painting it became clear that transfer of small amounts of murine chromatin is

ubiquitous. In the bizarre neoplastic recipient karyotype containing extreme fragmentation and an incidence of double minutes, it is almost impossible to identify these small fragments of murine chromatin by simple karyotyping, and this illustrates the power of *in-situ* technology. This technique was further supported with great convenience and sensitivity by murine IRS-PCR which produced a characteristic and unique murine fingerprint for each MHC fusion event. Using this technique, one could determine if within a particular fusion experiment the clones were derived from the same or independent fusion events. The answer was almost invariably a single fusion event with several clones dispersed by trypsinisation prior to selection. Clonal evolution of the human transgene but not the mouse transgene was observed which correlated with changes in components of the neoplastic phenotype.

In keeping with the clinical observation of high rates of LOH on chromosome 11 in the absence of 11p and 11q LOH concordance, and also with the observation of extreme re-arrangement of chromosome 11 in OVCAR3, it was not surprising to find fragmentation of the donated chromosome 11 in the MHCs. Indeed, the majority of hybrids contained fragmentation of chromosome 11 and these events included MHCs derived from the same fusion event with identical murine IRS-PCR fingerprints yet obvious differences due to fragmentation of the donated chromosome 11 by LOH analysis. Fragmentation of 11p13 and 11q24 occurred together frequently, dramatically observed for one clone (11OH1.2) which had a completely different neoplastic reverted phenotype despite an identical murine IRS-PCR fingerprint to another clone (11OH1.3) which retained the entire chromosome 11 and a suppressed, invasion-inhibited phenotype. It is interesting to note that the most significant concordant losses between MSPs on chromosome 11 in the clinical LOH studies were for D11S935 and D11S912, from the same region. The basis for this observed fragmentation and the same observation in clinical samples is unclear but may be due to two co-

ordinately inactivated tumour suppressor genes. The generation of OH3 as a clonal derivative of OVCAR3 whilst essential prior to microcell fusion, was nevertheless problematic because this sub-clone proved to be extremely sluggish at subcutaneous xenograft growth. Changing from nude to SCID mice, and manipulation of the matrigel and oestradiol environment did result in repeatable conditions for xenograft growth. One successfully generated xenograft was taken back into cell culture and the cell line OHX was developed from it. This line only required one fifth the number of cells to form a tumour and therefore further microcell fusions were performed using OHX as a recipient.

### 6.3.2 Identification of three functional loci.

Functional analysis provided unambiguous evidence for suppressor function associated with the introduction of chromosome 11 and 12. Furthermore, there is some evidence that two separable functions are located on chromosome 11.

It is difficult to interpret the chromosome 1 experiments. Several senescence functions are known to be located on chromosome 1 and the chromosome 1 donor that we used has been used previously by Carl Barrett's group to assess senescence (Sugawara et al, 1990). I was unable to extract an adequate amount of DNA from these clones for PCR so I cannot confirm that microcell transfer actually occurred.

Microsatellite analysis of 11p was hampered by the unexpectedly large number of uninformative markers found (i.e. the alleles for 556.1.5 and OH3 being identical). This means that information is lost about potential tumour suppressors and this is relevant since there are translocation breakpoints at 11p15.5 and 11q12-q13 in OH3 (Andy Walker, personal communication)

Clear *in-vitro* growth suppression occurred for all chromosome 11 microcell hybrids except those with fragmentation/deletion of D11S533, D11S1358 and telomeric 11p suggesting that the growth

suppressor could tentatively be assigned to these intervals. In particular, clone 11OHX2.3 which appeared to have lost all informative markers on the short arm of the donated chromosome grew particularly briskly.

Cell cycle analysis revealed no evidence of accumulation of the MHCs in any particular phase as compared with parental controls. This precluded an obvious effect of a cell cycle regulator. Furthermore FITC labelled Annexin-V binding was not elevated in the growth suppressed MHCs, implying that this growth suppression effect was not due to induction of apoptosis. *In-vivo* tumorigenicity (in SCID mice, with matrigel and oestradiol) was not completely suppressed, but tumour growth was delayed. Under conditions of high oestradiol dose, however, tumour growth could be completely suppressed, evidence in itself of the arbitrary nature of the subcutaneous xenograft assay.

Closer inspection of the growth suppressed cell lines revealed two distinct morphological patterns (figure 5.7). In two clones (11OH1.1 and 11OHX1.1) distal 11q was retained and a dramatic effect was observed. The cells grew in tight, clustered colonies and did not spread and migrate to occupy the available surface on the tissue culture dish. In comparison, the parent and control cell lines and MHCs containing chromosome 11 with deletions in 11q24 as small as 5.5 Mb grew with the same spread-out characteristics as the parent regardless of their *in-vitro* growth rates. For 11OH1.1 and 11OHX1.1, the self-imposed lack of spreading/migration combined with continued cellular proliferation resulted in colonies with apparently very small cells reminiscent of over-confluent cell cultures, which in three dimensional reality probably represent rounded up cells. In order to begin to understand this phenomenon, functional assays which address these phenotypes were employed.

### 6.3.3 The 11q invasion suppressor: dissecting the components involved

The clone containing a whole copy of chromosome 11 (11OH1.1) was investigated in more detail. Within 4 passages of the cell line being developed, the clustered phenotype described above had begun to revert to the parental spread-out phenotype. Two clonal sub-lines were developed from 11OH1.1 (called 11OH1.2 and 11OH1.3). These clones had identical murine PCR fingerprints implying that they were closely related but exhibited dramatically different *in-vitro* phenotypes. 11OH1.3 retained the original clustered appearance of 11OH1.1 and microsatellite mapping demonstrated retention of all transferred chromosome 11 loci examined. 11OH1.2 had deletions of the transferred chromosome 11 at D11S935 (11p13) and a 5.5 Mb deletion centred around D11S910 (11q24-q25) which overlaps with the distal LOH region defined in the ovarian cancer patients described above.

11OH1.3 was significantly inhibited (to about 37%) in its ability to invade into a matrigel layer compared with controls (and clones with deletion of the 11q24 locus including 11OH1.2). The functional effect of disrupting this locus is therefore a substantial increase in invasiveness, and it is noted at this point that the clinical phenotype which preceded these experiments was one of advanced stage, i.e. disseminated nodules invading widely over the surface of the peritoneum resulting in poor prognosis.

In order to understand which component of invasiveness was involved, dissection of the invasion assay into the component assays of tumour cell attachment and migration were performed. The effect of the basement membrane components laminin and collagen IV, and the plasma protein fibronectin (which is also a component of the extra-cellular matrix) were observed on migration and attachment of parent cells and MHCs.

#### *6.3.3.1 Tumour cell attachment*

A striking and specific effect of inhibition of laminin mediated attachment was observed which could be ascribed to the 11q24 locus

both quantitatively and qualitatively. In addition to integrins mediating attachment to laminin, a specific laminin receptor (32/67 kD laminin binding protein, or 67 LR) has been described and is upregulated in carcinomas (Liotta et al, 1984; Wewer et al, 1986). Laminin receptor has also been specifically examined in the OVCAR3 ovarian cancer cell line (Liebman et al, 1993). Sub-lines of OVCAR3 which form ascites/advanced disease have high expression of 67LR and low levels of expression of basement membrane components/collagenase IV. The converse is true for OVCAR3 cells in prolonged *in-vitro* culture or those that form subcutaneous tumours but not intra-peritoneal tumours. Over-expression of the laminin receptor has been shown to correlate with an adverse prognosis in lung and breast cancer. At least 5 integrins mediate attachment to laminin including $\alpha 2\beta 1$ (Languino et al, 1989; Maemura et al, 1995) and $\alpha 6\beta 4$ (Lee et al, 1992). The morphological alterations associated with introduction of the 11q24 locus are not dissimilar to the phenomenon of compaction whereby cells develop extensive and intimate contacts along their surfaces resulting in tightly adherent polarised epithelial sheets. These effects may be mediated by cadherins, catenins or the actin cytoskeleton (Reviewed in Gumbiner 1996), but it may simply be that inhibition of integrin pathways that ensure cell spreading on extra-cellular surfaces in the absence of down-regulation of cadherins and catenin results in default compaction due to a shift in balance between catenin/cadherin expression on the one hand and integrin expression on the other. This, of course, is purely speculative, but is worth mentioning.

#### *6.3.3.2 Tumour cell migration*

A further distinct effect was observed in addition to laminin attachment-inhibition. Clone 11OH1.2 with the 5.5 Mb deletion in 11q24 showed distinct stimulation of migration in response to a haptotactic collagen IV or fibronectin signal emanating from the under-surface of the transwell. Curiously, no such stimulation of

migration was seen in response to a laminin signal. In clone 11OH1.3, with the 11q region intact, clear abrogation of the collagen IV/fibronectin migratory stimulation phenomenon is observed suggesting that a pleiotropic process is co-ordinately inhibited in association with the introduction of the 11q24 region into OVCAR3.

### 6.4 Hypothesis

An 8.5 Mb region located in 11q24, when disrupted in primary tumours from patients with ovarian cancer, is associated with advanced stage (with widespread peritoneal dissemination of tumour) and poor prognosis. Introduction of chromosome 11 into a poorly differentiated ovarian cancer cell line (OVCAR3) with extensive re-arrangement of chromosome 11 mediates two distinct suppression phenotypes. One of these is growth inhibition and the function maps in the region between 11pter-q14. The other phenotype is inhibition of tumour cell invasiveness, which maps to a 5.5 Mb region in the microcell hybrids and overlaps over 4.5 Mb with the 8.5 Mb region described above (see figure 6.4). The region described independently by Davis et al (1996) in ovarian cancer also spans this 4.5 Mb region, confirming the LOH analysis. The invasion inhibited phenotype can be shown to be inhibited for both laminin mediated attachment and fibronectin/collagen IV stimulated migration suggesting that the 11q24 region may mediate suppression of a pleiotropic, co-ordinated and complex process of tumour cell invasion (figure 6.5).

Figure 6.4 **Consensus localisation data of the 11q24 region**

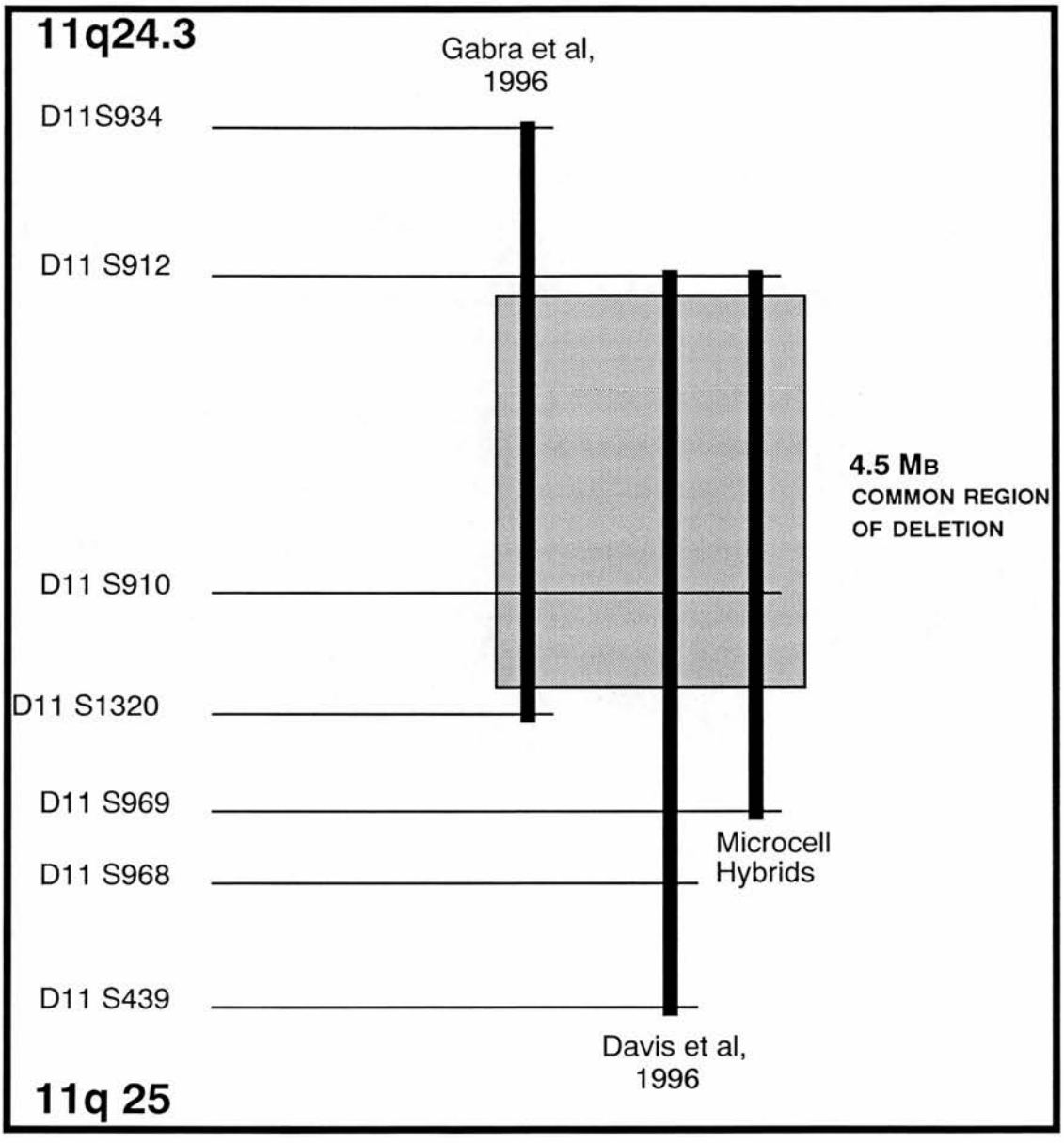
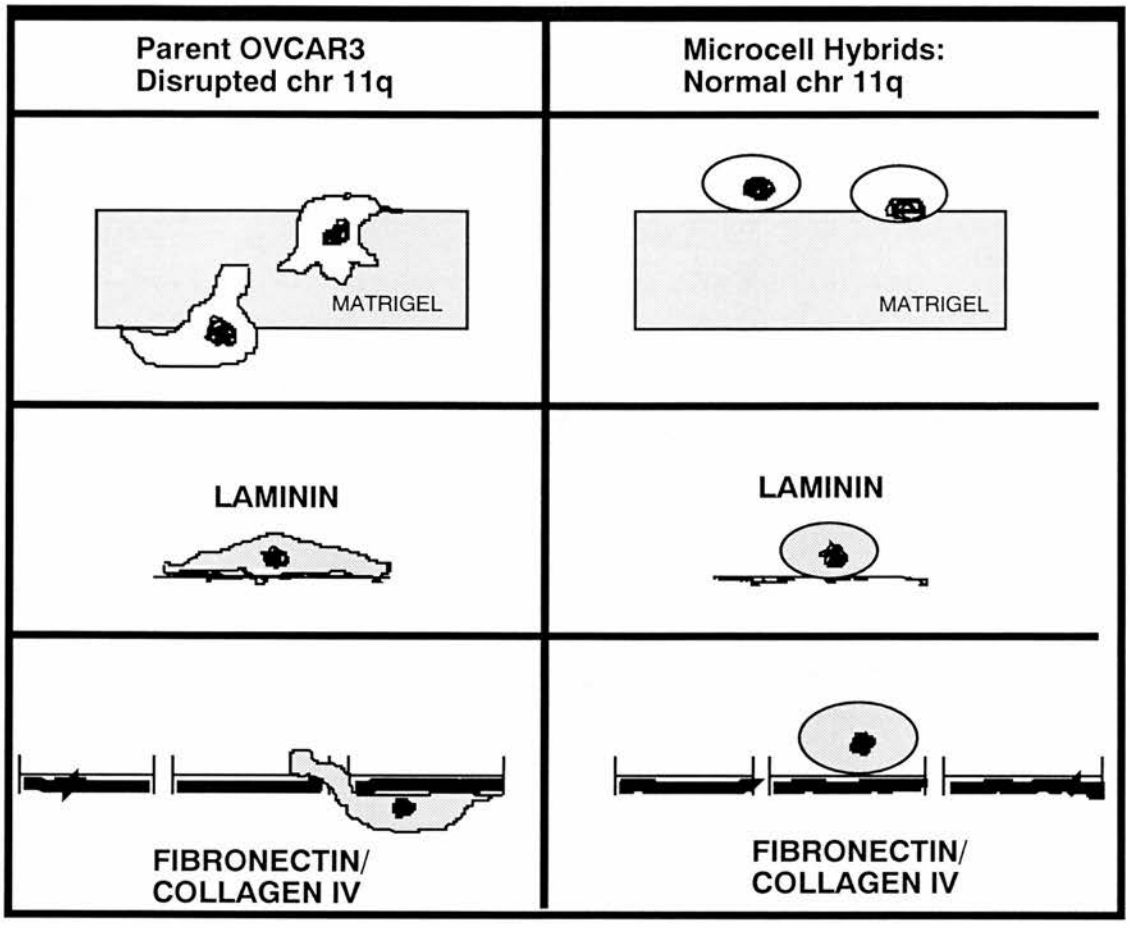


Figure 6.5 Phenotypic effects ascribed to the 11q24 locus



### 6.5 Summary and future directions

This study has succeeded in taking the adverse clinical phenotype of poor actuarial survival and developing an association between this and LOH at distal 11q. By a functional approach utilising somatic cell genetics, an underlying cell biological basis for this adverse phenotype has been defined, that of enhanced invasiveness in association with loss of this region. The matrigel invasion assay has been further dissected into its component parts, a specific cell adhesion/spreading effect mediated through laminin binding; and a cell migratory effect mediated by collagen IV / fibronectin haptotactic signals.

Further immunofluorescent analysis of integrins may allow the definition of some of the downstream components of this pleiotropic response associated with the introduction of distal 11q. Analysis of the expression of α2, α3, α6, β1 and β4, as well as catenins and cadherins may further define the basis of these phenotypic differences.

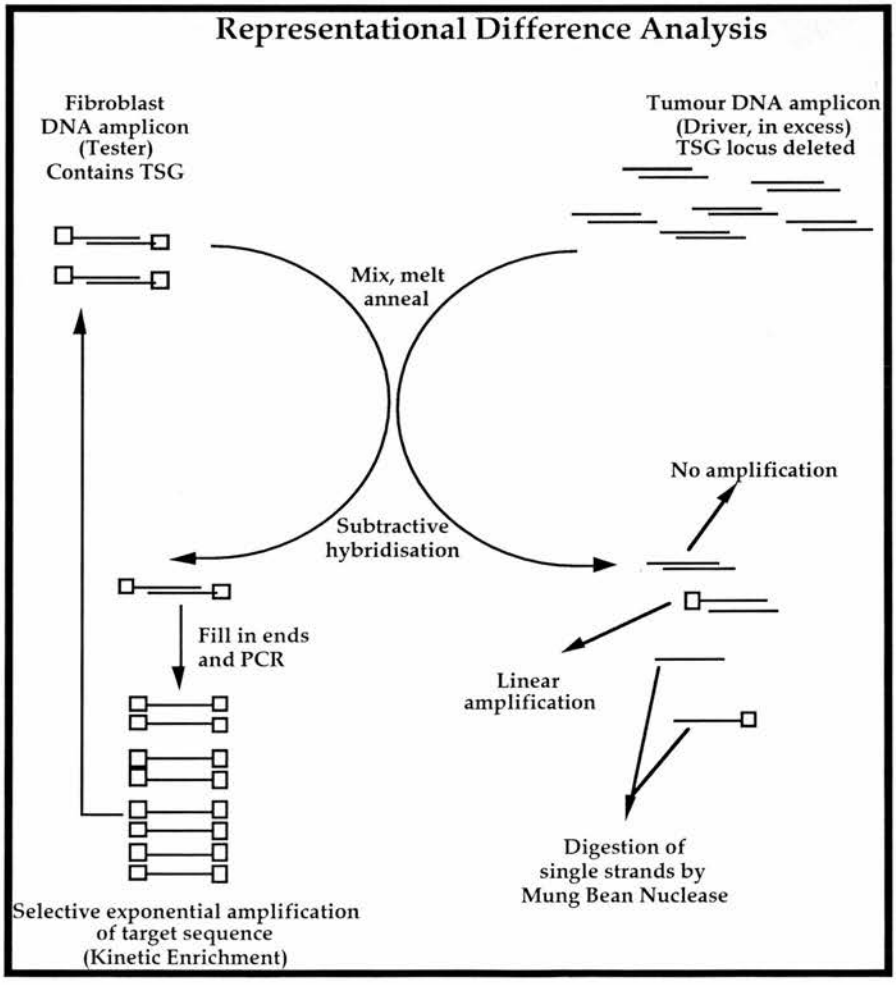
Further identification of chromosome 11 candidate genes may give some indication whether these genes may have some role to play in these phenotypes. In particular, genes within the 11q24 region (FLI1, ETS1, SRPR, FLI1, ZNF1 and NFRKB) may be involved, although they do not represent logical candidates based on their known functions. Known tumour suppressor genes on chromosome 11 (p57 at 11p15.5, WT1 at 11p13, KAI1 at 11p12 and NCAM at 11q22) may have some role, although microsatellite analysis suggests that they are not involved.

**An integrated positional-functional approach to cloning the 11q invasion-suppressor**

A YAC-contig spanning the consensus region of deletion in figure 6.4 is already available from the Human Genome Mapping Project and will be constructed for a positional cloning approach. However, at 4.5 Mb it is a little large for a small group such as ours and therefore it will be used in conjunction with markers derived from a difference cloning approach (see below).

Representational difference analysis (RDA) is an extremely powerful technique which allows anonymous sequences to be cloned from two closely related genetic complements with relatively few differences. It was developed by Nikolai Lisitsyn (Lisitsyn et al, 1993) and represents a major advance in difference cloning technology. In summary, representations of the genome are made by digestion with a 6 cutter restriction endo-nuclease and then PCR amplification in order to simplify the complexity of the genome and increase the amount of DNA available. Generating a representation improves the completeness of subsequent subtractive hybridisation. The genomic resource lacking the gene of interest is mixed in 80 fold excess (driver) with the resource containing the gene of interest which has had adapter ligated its ends (tester) (figure 6.6).

**Figure 6.6 Principles of Representational Difference Analysis (RDA)**



The samples are mixed, heated and re-annealed resulting in subtractive hybridisation of sequences common to both driver and tester. The only sequences which find other sequences with the ligated adapter are sequences unique to the tester representation. These are then selectively exponentially amplified by PCR. The entire process is repeated 3 times resulting in enrichment of the target by a factor of at least $10^6$. The resultant sequences are cloned into bluescript and can be used to isolate genes encoding phenotypic differences. We have established RDA in our laboratory and demonstrated that it can be used to clone the contents of a homozygous deletion found in an ovarian cancer cell line (Watson et al 1996). The application of RDA to the resources described in this thesis relate to its adaptation to cloning the difference between 2 closely related cDNA populations (Hubank and Schatz, 1994). Essentially identical conditions are employed for cDNA-RDA except that a 4-cutter is utilised instead of a 6-cutter since cDNA populations intrinsically are representations of the genome. Explicitly, the cDNA population from 11OH1.2 with an invasive phenotype and a 5.5 Mb deletion at 11q24 will be used as the driver and cDNA from 11OH1.3 retaining 11q24 intact will be used as the tester. Since they have identical murine IRS-PCR fingerprints, I would not expect to clone murine sequences from this experiment. This technique will, of course, not be successful if expression of the gene of interest is not deleted or structurally disrupted in a major way. Isolation of cDNA-RDA clones from this region will be the next major step in this project and will provide the link to identification of the structure of this gene and its biological role in ovarian cancer.

This thesis has taken the clinical phenotype of adverse prognosis and developed an integrated positional/functional strategy to identify genes responsible, when disrupted, for this adverse phenotype. The strategy allows an approach to a previously difficult problem in somatic cell genetics of cancer, that of how to proceed from a phenotype back to the gene. What is clear is that this process, though

demanding technically and in terms of interpretation, provides a real route to cloning clinically relevant tumour-suppressor genes in ovarian cancer.

# 7. REFERENCES

Adlercreutz, H.,Mousavi, Y.,Clark, J.,Hockerstedt, K.,Hamalainen, E.,Wahala, K.,Makela, T., and Hase, T. (1992). Dietary phytoestrogens and cancer: in vitro and in vivo studies. J Steroid Biochem Mol Biol, 41, 331-337.

Adlercreutz, H.,Bannwart, C.,Wahala, K.,Makela, T.,Brunow, G.,Hase, T.,Arosemena, P. J.,Kellis, J., Jr., and Vickery, L. E. (1993). Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Mol Biol, 44, 147-153.

Ahamed, S.,Wang, T. H.,Foster, J. S.,Bukovsky, A., and Wimalasena, J. (1996). Estradiol activates cyclin/Cdks in ovarian cancer cells (abstract 1488). Proc Am Assoc Cancer Res, 37 (pp. 218). Washington.

Akao, Y.,Seto, M.,Yamamoto, K.,Iida, S.,Nakazawa, S.,Inazawa, J.,Abe, T.,Takahashi, T., and Ueda, R. (1992). The rck gene associated with t(11-14) translocation is distinct from the mll/all-1 gene with t(4-11) and t(11-19) translocations. Cancer Res, 52, 6083-6087.

Al-Azraqi, A.,Chapman, C.,Challen, C.,Aswad, S.,Sinha, D.,Calvert, A. H., and Lunec, J. (1995). P53 mutations in primary human ovarian cancer as a determinant of resistance to carboplatin (meeting abstract). Proc Am Assoc Cancer Res, 36 (pp. 228).

Al-Azraqi, A.,Challen, C.,McKenna, D.,George, M.,Ghazal-Awad, S.,Angus, B.,Sinha, D.,Calvert, A. H., and Lunec, J. (1996). p21-WAF1 expressionand p53 status as determinants of response to carboplatin in primary human ovarian cancer.(abstract 1334). Proc Am Assoc Cancer Res, 37 (pp. 195). Washington.

Alama, A.,Barbieri, F.,Cagnoli, M.,Pedulla, F., and Ragni, N. (1996). Cyclin D1 expression correlates with malignancy in ovarian tumors (abstract 1403). Proc Am Assoc Cancer Res, 37 (pp. 205). Washington.

Albini, A.,Iwamoto, Y.,Kleinman, H. K.,Martin, G. R.,Aaronson, S. A.,Kozlowski, J. M., and McEwan, R. N. (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res, 47, 3239-3245.

Ali, I. U.,Lidereau, R.,Theillet, C., and Callahan, R. (1987). Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science, 238, 185-188.

Anderson, M. J.,Fasching, C. L.,Xu, H. J.,Benedict, W. F., and Stanbridge, E. J. (1994). Chromosome 13 transfer provides evidence for regulation of RB1 protein expression. Genes Chromosom Cancer, 9, 251-260.

Aznavoorian, S.,Murphy, A. N.,Stetler-Stevenson, W. G., and Liotta, L. A. (1993). Molecular aspects of tumor cell invasion and metastasis. Cancer, 71, 1368-1383.

Baak, J. P.,Chan, K. K.,Stolk, J. G., and Kenemans, P. (1987). Prognostic factors in borderline and invasive ovarian tumors of the common epithelial type. Pathol Res Pract, 182, 755-774.

Bader, S. A.,Fasching, C.,Brodeur, G. M., and Stanbridge, E. J. (1991). Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ, 2, 245-255.

Baiocchi, G.,Kavanagh, J. J.,Talpaz, M.,Wharton, J. T.,Gutterman, J. U., and Kurzrock, R. (1991). Expression of the macrophage colony stimulating factor and its receptor in gynecologic malignancies. Cancer, 67, 990.

Banerjee, A.,Xu, H. J.,Hu, S. X.,Araujo, D.,Takahashi, R.,Stanbridge, E. J., and Benedict, W. F. (1992). Changes in growth and tumorigenicity following reconstitution of retinoblastoma gene-function in various human cancer cell-types by microcell transfer of chromosome-13. Cancer Res, 52, 6297-6304.

Barski, G.,Sorieul, S., and Cornefert, F. (1961). 'Hybrid' type cells in combined cultures of two different mammalian cell strains. J. Nat. Cancer Inst., 26, 1269-1291.

Bast, R., Jr.,Boyer, C. M.,Jacobs, I.,Xu, F. J.,Wu, S.,Wiener, J.,Kohler, M., and Berchuck, A. (1993). Cell growth regulation in epithelial ovarian cancer. Cancer, 71, 1597-1601.

Bell, D. A., and Scully, R. E. (1994). Early de-novo ovarian-carcinoma - a study of 14 cases. Cancer, 73, 1859-1864.

Bello, M. J., andRey, J. A. (1990). Chromosome aberrations in metastatic ovarian cancer: relationship with abnormalities in primary tumours. Int. J. Cancer, 45, 50-54.

Berchuck, A.,Kamel, A.,Whitaker, R.,Kerns, B.,Olt, G.,Kinney, R.,Soper, J. T.,Dodge, R.,Clarke-Pearson, D. L., and Marks, P. (1990). Overexpression of Her-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res, 50, 4087.

Berchuck, A.,Elbendary, A.,Havrilesky, L.,Rodriguez, G. C., and Bast, R. C. (1994). Pathogenesis of ovarian cancers. J Soc Gyn I, 1, 181-190.

Berube, N. G.,Speevak, M. D., and Chevrette, M. (1994). Suppression of tumorigenicity of human prostate cancer cells by introduction of human chromosome del(12)(q13). Cancer Res, 54, 3077-3081.

Bonte, J. (1979). Developments in endocrine related therapy of endometrial and ovarian cancer. Rev. Endocr. Rel. Cancer, 3, 11.

Bourne, T. H.,Whitehead, M. I.,Campbell, S.,Royston, P.,Bhan, V., and Collins, W. P. (1991). Ultrasound screening for familial ovarian cancer. Gynecol Oncol, 43, 92-97.

Bressac, B.,Kew, M.,Wands, J., and Ozturk, M. (1991). Selective G-mutation to T-mutation of the p53 gene in hepatocellular carcinoma in Southern Africa. Nature, 350, 429-431.

Bronner, C. E.,Baker, S. M.,Morrison, P. T.,Warren, G.,Smith, L. G.,Lescoe, M. K.,Kane, M.,Earabino, C.,Lipford, J.,Lindblom, A., and et, a. (1994). Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature, 368, 258-261.

Bruening, W.,Gros, P.,Sato, T.,Stanimir, J.,Nakamura, Y.,Housman, D., and Pelletier, J. (1993). Analysis of the 11p13 wilms-tumor suppressor gene (wt1) in ovarian-tumors. Cancer Invest, 11, 393-399.

Byers, T.,Marshall, J.,Graham, S.,Mettlin, C., and Swanson, M. (1983). A case-control study of dietary and nondietary factors in ovarian cancer. J Natl Cancer Inst, 71, 681-686.

Call, K. M.,Glaser, T.,Ito, C. Y.,Buckler, A. J.,Pelletier, J.,Haber, D. A.,Rose, E. A.,Kral, A.,Yeger, H.,Lewis, W. H., and et, a. (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell, 60, 509-520.

Cao, Q.,Cedrone, E.,Barrett, C., and Wang, N. (1993). Suppression of in-vitro growth of ovarian-carcinoma cells by microcell-mediated chromosome 11 transfer. Am J Hum Genet, 53, 1517.

Cao, O.,Cedrone, E.,Keng, P.,Barrett, C., and Wang, N. (1995). Study of tumor suppression in human ovarian-carcinoma cells skoy-3 by microcell-mediated chromosome transfer. Am J Hum Genet, 57, 233.

Carson, D.A. and Lois, A. (1995). Cancer Progression and p53. Lancet, 346, 1009-1011.

Carter, S. L.,Negrini, M.,Baffa, R.,Gillum, D. R.,Rosenberg, A. L.,Schwartz, G. F., and Croce, C. M. (1994). Loss of Heterozygosity at 11q22-q23 in breast cancer. Cancer Res, 54, 6270-6274.

Casey, G.,Plummer, S.,Hoeltge, G.,Scanlon, D.,Fasching, C., and Stanbridge, E. J. (1993). Functional evidence for a breast-cancer growth suppressor gene on chromosome-17. Hum Mol Genet, 2, 1921-1927.

Chambers, S. K.,Wang, Y. X.,Gertz, R. E., and Kacinski, B. M. (1995). Macrophage-colony-stimulating factor mediates invasion of ovarian-cancer cells through urokinase. Cancer Res, 55, 1578-1585.

Chang, J.,Bridgewater, J.,Gore, M.,Fisher, C.,Schofield, J.,A'Hern, R.,Ponder, B.,Jacobs, I.,McKeage, M.,Kelland, L., and Harap, K. (1994). Non-surgical aspects of ovarian cancer. Lancet, 343, 335-341.

Chen, P.,Lin, H. H., and Weissman, B. E. (1995). A functional analysis of tumor suppressor activity for peripheral neuroepitheliomas by monochromosome transfer. Oncogene, 10, 577-586.

Chien, C. H., andChow, S. N. (1993). Point mutation of the ras oncogene in human ovarian-cancer. Dna Cell Biol, 12, 623-627.

Cliby, W.,Ritland, S.,Hartmann, L.,Dodson, M.,Halling, K. C.,Keeney, G.,Podratz, K. C., and Jenkins, R. B. (1993). Human epithelial ovarian-cancer allelotype. Cancer Res, 53, 2393-2398.

Clurman, B. E., andRoberts, J. M. (1995). Cell cycle and cancer. J Natl Cancer Inst, 87, 149.

Cobrinik, D.,Dowdy, S. F.,Hinds, P. W.,Mittnacht, S., and Weinberg, R. A. (1992). The retinoblastoma protein and regulation of cell cycling. TIBS, 17, 132.

Collier, I. E.,Smith, J.,Kronberger, A.,Bauer, E. A.,Wilhelm, S. M.,Eisen, A. Z., and Goldberg, G. I. (1988). The structure of the human skin fibroblast collagenase gene. J Biol Chem, 263, 10711-10713.

Conway, K.,Morgan, D.,Phillips, K. K.,Yuspa, S. H., and Weissman, B. E. (1992). Tumorigenic suppression of a human cutaneous squamous-cell carcinoma cell-line in the nude-mouse skin-graft assay. Cancer Res, 52, 6487-6495.

Cornelis, R. S.,Neuhausen, S. L., and Johannson, O. (1995). High allele loss rates at 17q12-21 in breast and ovarian tumors from BRCA1-linked families. Genes Chrom Cancer, 13, 203.

Couch, F.J., Farid, L.M., Deshano, M.L., Tavtigian, S.V., Calzone, K., Campeau, L.,Peng, Y.,Bogden, B.,Chen, Q.,Neuhausen, S.,Shattuckeidens, D.,Godwin, A.K.,Daly, M.,Radford, D. M.,Sedlacek, S.,Rommens, J.,Simard, J.,Garber, J.,Merajver, S., and Weber, B. L. (1996). BRCA2 germline mutations in male breast-cancer cases and breast-cancer families. Nature Genet, 13, 123-125.

Couillin, P.,Leguern, E.,Vignal, A.,Fizames, C.,Ravise N.,Delportes, D.,Reguigne,I. Rosier, M.,Junien, C.,Van-Heyningen, V., and Weissenbach, J. (1994). Assignment of 112 microsatellite markers to 23 chromosome-11 subregions delineated by somatic hybrids - comparison with the genetic-map. Genomics, 21, 379-387.

Cramer, D. W.,Welch, W. R.,Scully, R. E., and Wojciechowski, C. A. (1982a). Ovarian cancer and talc: a case-control study. Cancer, 50, 372-376.

Cramer, D. W.,Hutchison, G. B.,Welch, W. R.,Scully, R. E., and Knapp, R. C. (1982b). Factors affecting the association of oral contraceptives and ovarian cancer. N Engl J Med, 307, 1047-1051.

Cramer, D. W.,Hutchison, G. B.,Welch, W. R.,Scully, R. E., and Ryan, K. J. (1983). Determinants of ovarian cancer risk I. Reproductive experiences and family history. J Natl Cancer Inst, 71, 711-716.

Cramer, D. W.,Welch, W. R.,Hutchison, G. B.,Willett, W., and Scully, R. E. (1984). Dietary animal fat in relation to ovarian cancer risk. Obstet Gynecol, 63, 833-838.

Cramer, D. W.,Harlow, B. L.,Willett, W. C.,Welch, W. R.,Bell, D. A.,Scully, R. E.,Ng, W. G., and Knapp, R. C. (1989). Galactose consumption and metabolism in relation to the risk of ovarian cancer. Lancet, 2, 66-71.

Cramer, D. W. (1990). Epidemiologic aspects of early menopause and ovarian cancer. Ann N Y Acad Sci, 592, 363-375.

Cuthbert, A. P.,Trott, D. A.,Ekong, R. M.,Jezzard, S.,England, N. L.,Themis, M.,Todd, C. M., and Newbold, R. F. (1995). Construction and characterization of a highly stable human-rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenet Cell Genet, 71, 68-76.

Davis, M.,Hitchcock, A.,Foulkes, W. D., and Campbell, I. G. (1996). Refinement of two chromosome 11q regions of loss o heterozygosity in ovarian cancer. Cancer Res, 56, 741-744.

de-Souza, P. L., andFriedlander, M. L. (1992). Prognostic factors in ovarian cancer. Hematol Oncol Clin North Am, 6, 761-782.

Deshane, J.,Loechel, F.,Conry, R. M.,Siegal, G. P.,King, C. R., and Curiel, D. T. (1994). Intracellular single-chain antibody-directed against erbb2 down-regulates cell-surface erbb2 and exhibits a selective antiproliferative effect in erbb2 overexpressing cancer cell-lines. Gene Ther, 1, 332-337.

Dong, J. T.,Lamb, P. W.,Rinkerschaeffer, C. W.,Vukanovic, J.,Ichikawa, T.,Isaacs, J. T., and Barrett, J. C. (1995). Kai1, a metastasis suppressor gene for prostate-cancer on human-chromosome 11p11.2. Science, 268, 884-886.

Dowdy, S. F.,Scanlon, D. J.,Fasching, C. L.,Casey, G., and Stanbridge, E. J. (1990). Irradiation microcell-mediated chromosome transfer (XMMCT): the generation of specific chromosomal arm deletions. Genes Chromosom Cancer, 2, 318-327.

Dowdy, S. F.,Lai, K. M.,Weissman, B. E.,Matsui, Y.,Hogan, B. L., and Stanbridge, E. J. (1991). The isolation and characterization of a novel cDNA demonstrating an altered mRNA level in nontumorigenic Wilms' microcell hybrid cells. Nucleic Acids Res, 19, 5763-5769.

Easton, D. F.,Bishop, D. T.,Ford, D.,Crockford, G. P., and Consortium, t. B. C. L. (1993a). Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. Am J Human Genet, 52, 678-701.

Easton, D.,Ford, D., and Peto, J. (1993b). Inherited susceptibility to breast cancer. Cancer Surveys, 18, 95.

Eccles, D.,Cranston, G.,Steel, C. M.,Nakamura, Y., and Leonard, R. C. F. (1990). Allele losses on chromosome 17 in human epithelial ovarian cancer. Oncogene, 5, 1599-1601.

Eccles, D. M.,Russell, S.,Haites, N. E.,Atkinson, R.,Bell, D. W.,Gruber, L.,Hickey, I.,Kelly, K.,Kitchener, H., and Leonard, R. (1992a). Early loss of heterozygosity on 17q in ovarian-cancer. Oncogene, 7, 2069-2072.

Eccles, D. M.,Gruber, L.,Stewart, M.,Steel, C. M., and Leonard-RCF (1992b). Allele loss on chromosome 11p is associated with poor survival in ovarian cancer. Dis Markers, 10, 95-99.

Ege, T., andRingertz, N. R. (1974). Preparation of microcells by enucleation of micronucleate cells. Exp Cell Res, 87, 378-382.

Ehlen, T., and Dubeau, L. (1990). Loss of heterozygosity on chromosomal segments 3p, 6q and 11p in human ovarian cancer. Oncogene, 5, 219-223.

Elledge, R.M. and Lee, W.-H (1995). Life and Death by p53. BioEssays, 17, 923-930.

Elices, M. J., andHemler, M. E. (1989). The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc Natl Acad Sci U S A, 86, 9906-9910.

Eva, A.,Robbins, K.C.,Andersen, P.R.,Srinivasan, A.,Tronick, S.R.,Reddy, E.P.,Ellmore, N.W.,Galen, A.T.,Lautenberger, J.A.,Papas, T.S.,Westin, E.H.,Wong-Staal, F.,Gallo, R.C., and Aaronson, S.A. (1982). Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. Nature, 295, 116-119.

Ewing, C. M.,Ru, N.,Morton, R. A.,Robinson, J. C.,Wheelock, M. J.,Johnson, K. R.,Barrett, J. C., and Isaacs, W. B. (1995). Chromosome-5 suppresses tumorigenicity of pc3 prostate-cancer cells - correlation with reexpression of alpha-catenin and restoration of e-cadherin function. Cancer Res, 55, 4813-4817.

Falcioni, R.,Cimino, L.,Gentileschi, M. P.,D'Agnano, I.,Zupi, G.,Kennel, S. J., and Sacchi, A. (1994). Expression of beta 1, beta 3, beta 4, and beta 5 integrins by human lung carcinoma cells of different histotypes. Exp Cell Res, 210, 113-122.

Fathalla, M. F. (1971). Incessant ovulation- a factor in ovarian neoplasia? Lancet, II, 163.

Fearon, E. R.,Vogelstein, B., and Feinberg, A. P. (1984). Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumours. Nature, 309, 176-178.

Fearon, E. R.,Feinberg, A. P.,Hamilton, S. H., and Vogelstein, B. (1985). Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature, 318, 377-380.

Felch, M. E.,Willis, R. A.,Penney, D. P.,Keng, P. C., and Phipps, R. P. (1992). Expression of alpha 6 beta 1 integrin, the laminin receptor, on subsets of normal murine lung fibroblasts and its upregulation by the inflammatory cytokines IFN-gamma and TNF-alpha. Reg Immunol, 4, 363-370.

Ferrari, E.,Maffeo, D. A.,Graziano, R.,Gallo, M. S.,Pignata, S.,De-Rosa, L.,Montella, M., and Pergola, M. (1994). Intraperitoneal chemotherapy with carboplatin and recombinant interferon alpha in ovarian cancer. Eur J Gynaecol Oncol, 15, 437-442.

FIGO Staging Committee. (1986). Staging Announcement. Gynecol Oncol, 25, 383.

Filmus, J. E., andBuick, R. N. (1985). Stability of c-K-ras amplification during progression in a patient with adenocarcinoma of the ovary. Cancer Res, 45, 4468.

Fishel, R.,Lescoe, M. K.,Rao, M. R.,Copeland, N. G.,Jenkins, N. A.,Garber, J.,Kane, M., and Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer [published erratum appears in Cell 1994 Apr 8;77(1):167]. Cell, 75, 1027-1038.

Ford, D., andEaston, D. F. (1995). The genetics of breast and ovarian cancer. Br J Cancer, 72, 805.

Foulkes, W. D.,Black, D. M.,Stamp, G. W. H.,Solomon, E., and Trowsdale, J. (1993). Very frequent loss of heterozygosity throughout chromosome-17 in sporadic ovarian-carcinoma. Int J Cancer, 54, 220-225.

Foulkes, W. D.,Campbell, I. G.,Stamp, G., and Trowsdale, J. (1993). Loss of heterozygosity and amplification on chromosome-11q in human ovarian-cancer. Br J Cancer, 67, 268-273.

Foulkes, W. D.,Stamp, G. W. H.,Afzal, S.,Lalani, N.,McFarlane, C. P.,Trowsdale, J., and Campbell, I. G. (1995). MDM2 overexpression is rare in ovarian carcinoma irrespective of p53 mutation status. Br J Cancer, 72, 883.

Fournier, R. E. K., andRuddle, F. H. (1977). Microcell-mediated transfer of murine chromosomes into mouse, Chinese Hamster, and human somatic cells. Proc. Natl. Acad. Sci. USA, 74, 319-323.

Frank, T. S.,Bartos, R. E.,Haefner, H. K.,Roberts, J. A.,Wilson, M. D., and Hubbell, G. P. (1994). Loss of heterozygosity and overexpression of the p53 gene in ovarian-carcinoma. Mod Pathol, 7, 3-8.

Friedlander, M. L.,Hedley, D. W.,Taylor, I. W.,Russell, P.,Coates, A. S., and Tattersall, M. H. (1984). Influence of cellular DNA content on survival in advanced ovarian cancer. Cancer Res, 44, 397-400.

Friedlander, M. L.,Hedley, D. W.,Swanson, C., and Russell, P. (1988). Prediction of long-term survival by flow cytometric analysis of cellular DNA content in patients with advanced ovarian cancer. J Clin Oncol, 6, 282-290.

Friedman, M.,Lagios, M.,Markowwitz, A.,Jones, H.,Resser, K., and Hoffman, P. (1979). Estradiol (ER) and Progesterone receptors (PR) in ovarian cancer - clinical and pathological correlation. Clin Res, 27, 385A.

Fu, X., andHoffman, R. M. (1993). Human ovarian carcinoma metastatic models constructed in nude mice by orthotopic transplantation of histologically-intact patient specimens. Anticancer Res, 13, 283-286.

Fujimori, M.,Wells, S. A., and Nakamura, Y. (1992). Fine scale mapping of the gene responsible for multiple endocrine neoplasia type 1 (MEN1). Am. J. Hum. Genet., 50, 399-403.

Fuqua, S. A. W.,Hill, S. M.,Chamness, G. C.,Benedix, M. G.,Greene, G. L.,O'Malley, B. W., and McGuire, W. L. (1991a). Progesterone receptor gene restriction fragment length polymorphisms in human breast tumors. J Natl Cancer Inst, 83, 1157-1160.

Fuqua, S. A.,Fitzgerald, S. D.,Chamness, G. C.,Tandon, A. K.,McDonnell, D. P.,Nawaz, Z.,O'Malley, B. W., and McGuire, W. L. (1991b). Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res, 51, 105-109.

Fuqua, S. A.,Fitzgerald, S. D.,Allred, D. C.,Elledge, R. M.,Nawaz, Z.,McDonnell, D. P.,O'Malley, B. W.,Greene, G. L., and McGuire, W. L. (1992). Inhibition of estrogen receptor action by a naturally occuring variant in human breast tumors. Cancer Res, 52, 483-486.

Fuqua, S. A.,Allred, D. C.,Elledge, R. M.,Krieg, S. L.,Benedix, M. G.,Nawaz, Z.,O'Malley, B. W.,Greene, G. L., and McGuire, W. L. (1993). The Er-positive/PgR-negative breast cancer phenotype is not associated with mutations within the DNA binding domain. Breast Cancer Res Treat, 26, 191-202.

Futreal, P. A.,Liu, Q.,Shattuck-Eidens, D.,Cochran, C.,Harshman, K.,Tavtigian, S.,Bennett, L. M.,Haugen-Strano, A.,Swensen, J.,Miki, Y., and et, a. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. Science, 266, 120-2.

Gabra, H.,Taylor, L.,Cohen, B. B.,Lessels, A.,Eccles, D. M.,Leonard, R. C. F.,Smyth, J. F., and Steel, C. M. (1995a). Chromosome-11 allele imbalance and clinicopathological correlates in ovarian-tumors. Br J Cancer, 72, 367-375.

Gabra, H.,Langdon, S. P.,Watson, J. E. V.,Hawkins, R. A.,Cohen, B. B.,Taylor, L.,Mackay, J.,Steel, C. M.,Leonard, R. C. F., and Smyth, J. F. (1995b). Loss of heterozygosity at 11q22 correlates with low progesterone receptor content in epithelial ovarian cancer. Clin Cancer Res, 1, 945-953.

Gabra, H.,Watson, J. E. V.,Taylor, K. J.,MacKay, J.,Leonard, R. C. F.,Steel, C. M.,Porteous, D. J., and Smyth, J. F. (1996a). Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian-cancer associated with poor-prognosis. Cancer Res, 56, 950-954.

Gabra, H.,Watson, J. E. V.,Eccles, D. M.,Taylor, L.,Taylor, K. J.,Cohen, B. B.,Leonard, R. C. F.,Porteous, D. J.,Smyth, J. F., and Steel, C. M. (1996b). A statistical-analysis of chromosome-11 and chromosome-17 loss of heterozygosity in epithelial ovarian-cancer. Int J Oncol, 8, 625-631.

Gallion, H. H.,Powell, D. E.,Morrow, J. K.,Pieretti, M.,Case, E.,Turker, M. S.,DePriest, P. D.,Hunter, J. E., and Van-Nagell-JR, J. (1992). Molecular genetic changes in human epithelial ovarian malignancies. Gynecol Oncol, 47, 137-142.

Gardner, W. U. (1958). Further studies on experimental ovarian tumorigenesis. Proc Am Ass Cancer Res, 2, 300.

Gavrieli, Y.,Sherman, Y., and Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol, 119, 493-501.

Gayther, S.,Warren, W.,Mazoyer, S.,Russel, P. A.,Harrington, P. A.,Chiano, M.,Seal, S.,Hamoudi, R.,van Rensburg, E. J.,Dunning, A. M.,Love, R.,Evans, G.,Easton, D.,Clayton, D.,Stratton, M. R., and Ponder, B. A. J. (1995). Germline mutations in the BRCA1 gene in breast and ovarian cancer families provides evidence for a genotype-phenotype correlation. Nature Genet, 11, 428.

Gessler, M.,Poustka, A.,Cavenee, W.,Neve, R. L.,Orkin, S. H., and Bruns, G. A. (1990). Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature, 343, 774-778.

Giancotti, F. G., andRuoslahti, E. (1990). Elevated levels of the alpha 5 beta 1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell, 60, 849-859.

Goldin, B. R.,Woods, M. N.,Spiegelman, D. L.,Longcope, C.,Morrill-LaBrode, A.,Dwyer, J. T.,Gualtieri, L. J.,Hertzmark, E., and Gorbach, S. L. (1994). The effect of dietary fat and fiber on serum estrogen concentrations in premenopausal women under controlled dietary conditions. Cancer, 74, 1125-1131.

Gorczyca, W.,Tuziak, T.,Kram, A.,Melamed, M.R., and Darzynkiewicz, Z. (1994). Detection of apoptosis-associated DNA strand breaks in fine-needle aspiration biopsies by in-situ end labeling of fragmented DNA. Cytometry, 15, 169-175.

Goss, S. J., and Harris, H. (1975). New methods for mapping genes in human chromosomes. Nature, 255, 680-684.

Goyette, M. C.,Cho, K.,Fasching, C. L.,Levy, D. B.,Kinzler, K. W.,Paraskeva, C.,Vogelstein, B., and Stanbridge, E. J. (1992). Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. Mol Cell Biol, 12, 1387-1395.

Gudmundsson, J.,Barkardottir, R. B.,Eiriksdottir, G.,Baldursson, T.,Arason, A.,Egilsson, V., and Ingvarsson, S. (1995). Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. Br. J. Cancer, 72, 696-701.

Guthrie, D.,Davy, M. L., and Philips, P. R. (1984). A study of 656 patients with 'early' ovarian cancer. Gynecol Oncol, 17, 363-369.

Gwinn, M. L.,Webster, L. A.,Lee, N. C.,Layde, P. M., and Rubin, G. L. (1986). Alcohol consumption and ovarian cancer risk. Am J Epidemiol, 123, 759-766.

Gyapay, G.,Morissette, J.,Vignal, A.,Dib, C.,Fizames, C.,Millasseau, P.,Marc, S.,Bernardi, G.,Lathrop, M., and Weissenbach, J. (1994). The 1993-94 Genethon human genetic linkage map. Nature Genetics, 7, 246-300.

Haffner, R. and Oren, M. (1995). Biochemical properties and biological effects of p53. Current Biology, 5, 84-90.

Hall, J. M.,Friedman, L.,Guenther, C.,Lee, M. K.,Weber, J. L.,Black, D. M., and King, M.-C. (1992). Closing in on a breast cancer gene on chromosome 17q. Am J Hum Genet, 50, 1235-1342.

Hamilton, T. C.,Young, R. C.,McKoy, W. M.,Grotzinger, K. R.,Green, J. A.,Chu, E. W.,Whang-Peng, J.,Rogan, A. M.,Green, W. R., and Ozols, R. F. (1983). Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. Cancer Res, 43, 5379-5389.

Hampton, G. M.,Penny, L. A.,Baergen, R. N.,Larson, A.,Brewer, C.,Liao, S.,Busby-Earle, R. M. C.,Williams, A. W. R.,Steel, C. M.,Bird, C. C.,Stanbridge, E. J., and Evans, G. A. (1994). Loss of heterozygosity in cervical carcinoma: subchromosomal localisation of a putative tumor-suppressor gene to chromosome 11q22-q24. Proc Natl Acad Sci USA, 91, 6953-6957.

Hampton, G. M.,Mannermaa, A.,Winquist, R.,Alavaikko, M.,Blanco, G.,Taskinen, P. J.,Kiviniemi, H.,Newsham, I.,Cavenee, W. K., and Evans, G. A. (1994). Loss of Heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. Cancer Res, 54, 4586-4589.

Harlow, B. L.,Cramer, D. W.,Bell, D. A., and Welch, W. R. (1992). Perineal exposure to talc and ovarian cancer risk. Obstet Gynecol, 80, 19-26.

Harris, H., andWatkins, J. F. (1965). Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. Nature, 205, 640-646.

Harris, H.,Miller, O. J.,Klein, G.,Worst, P., and Tachibana, T. (1969). Suppression of malignancy by cell fusion. Nature, 223, 363-368.

Hart, I.R.andFidler, I.J. (1978). An in-vitro quantitative assay for tumour cell invasion. Cancer Res, 38, 3218-3224.

Heaps, J. M.,Nieberg, R. K., and Berek, J. S. (1990). Malignant neoplasms arising in endometriosis. Obstet Gynecol, 75, 1023-1028.

Henriksen, R.,Strang, P.,Wilander, E.,Backstrom, T.,Tribukait, B., and Oberg, K. (1994). P53 expression in epithelial ovarian neoplasms - relationship to clinical and pathological parameters, ki-67 expression and flow-cytometry. Gynecol Oncol, 53, 301-306.

Herrinton, L. J.,Stanford, J. L.,Schwartz, S. M., and Weiss, N. S. (1994). Ovarian cancer incidence among Asian migrants to the United States and their descendants. J Natl Cancer Inst, 86, 1336-1339.

Heutnik, P.,A.G.L., V. d. M.,L.A., S.,Van Gils, A. P. G.,Bardoel, A.,Breedveld, G. J.,Van Vliet, M.,Van Ommen, G.-J. B.,Cornellise, C. J.,Oostra, B. A.,Weber, J. L., and Devilee, P. (1992). A gene subject to imprinting and responsible for hereditary paraganglionomas maps to chromosome 11q23-qter. Hum. Mol. Genet., 1, 7-10.

Holt, J. A.,Waggoner, S. E.,Lee, E. Y.,Hubby, M. M., and Hamilton, T. C. (1987). Serum CA 125 and survival of mice inoculated with ovarian carcinoma and treated with antiestrogen, estrogen, or progestin. Gynecol Oncol, 27, 282-293.

Holt, J. T.,Thompson, M. E.,Szabo, C.,Robinsonbenion, C.,Arteaga, C. L.,King, M. C., and Jensen, R. A. (1996). Growth-retardation and tumor-inhibition by brca1. Nature Genet, 12, 298-302.

Horikawa, I.,Yamada, H.,Kugoh, H.,Yuasa, Y.,Suzuki, M., and Oshimura, M. (1995). Subchromosomal mapping of a putative transformation suppressor gene on human chromosome-1. Jpn J Cancer Res, 86, 444-450.

Horikawa, I.,Choi, C.,Cho, S.,Koi, M., and Barrett (1996). Positional and functional analyses of ovarian cancer suppressor genes on chromosome X (abstract 3763). In Proc Am Assoc Cancer Res, 37 (pp. 549). Washington.

Horwitz, K. B., andMcGuire, W. L. (1978). Estrogen control of progesterone receptor in human breast cancer: correlation with nuclear processing of estrogen receptor. J Biol Chem, 253, 2223-2228.

Hubank, M., andSchatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res, 22, 5640-5648.

Ichikawa, T.,Ichikawa, Y., and Isaacs, J. T. (1991). Genetic factors and metastatic potential of prostatic cancer. Cancer Surv, 11, 35-42.

Ichikawa, T.,Ichikawa, Y.,Dong, J.,Hawkins, A. L.,Griffin, C. A.,Isaacs, W. B.,Oshimura, M.,Barrett, J. C., and Isaacs, J. T. (1992). Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11. Cancer Res, 52, 3486-3490.

Ichikawa, T.,Nihei, N.,Suzuki, H.,Oshimura, M.,Emi, M.,Nakamura, Y.,Hayata, I.,Isaacs, J. T., and Shimazaki, J. (1994). Suppression of metastasis of rat prostatic cancer by introducing human chromosome 8. Cancer Res, 54, 2299-2302.

Imamura, H.,Takao, S., and Aikou, T. (1994). A modified invasion-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumor cell invasion. Cancer Res, 54, 3620-3624.

Iwabuchi, H.,Sakunaga, H.,Sakamoto, M.,Yang-Feng, T. L.,Pinkel, D., and Gray, J. W. (1995). Genetic progression model in ovarian cancer with comparative genomic hybridisation (meeting abstract). In Proc Am Assoc Cancer Res, 36 (pp. 226).

Iwabuchi, H.,Sakamoto, M.,Sakunaga, I I.,Ma, Y. Y.,Carcangiu, M. L.,Pinkel, D.,Yangfeng, T. L., and Gray, J. W. (1995). Genetic-analysis of benign, low-grade, and high-grade ovarian-tumors. Cancer Res, 55, 6172-6180.

Jaakkola, S.,Salmikangas, P.,Nylund, S.,Partanen, J.,Armstrong, E.,Pyrhonen, S.,Lehtovirta, P., and Nevanlinna, H. (1993). Amplification of FGFR4 gene in human breast and gynecological cancers. Int J Cancer, 54, 378-382.

Jabara, A. G. (1962). Induction of canine ovarian tumors by diethylstilbestrol and progesterone. Aust J Exp Biol, 40, 139-152.

James, M. R.,Richard III, C. W.,Schott, J.-J.,Youstry, C.,Clark, K.,Bell, J.,Terwilliger, J. D.,Hazan, J.,Dubay, C.,Vignal, A.,Agrapart, M.,Imai, T.,Nakamura, Y.,Polymeropoulos, M.,Weissenbach, J.,Cox, D. R., and Lathrop, G. M. (1994). A radiation hybrid map of 506 STS markers spanning human chromosome 11. Nature Genet., 8, 70-76.

Jenkins, R. B.,Bartelt, D.,Stalboerger, P.,Persons, D.,Dahl, R. J.,Podratz, K.,Keeney, G., and L., H. (1993). Cytogenetic studies of epithelial ovarian-carcinoma. Cancer Genet Cytogenet, 71, 76-86.

Jensen, R. A.,Thompson, M. E.,Jetton, T. L.,Szabo, C. I.,Vandermeer, R.,Helou, B.,Tronick, S. R.,Page, D. L.,King, M. C., and Holt, J. T. (1996). Brca1 is secreted and exhibits properties of a granin. Nature Genet, 12, 303-308.

Kamb, A. (1995). Cell-cycle regulators and cancer. Trends Genet, 11, 136.
Kao, F.-T.,Jones, C., and Puck, T. T. (1976). Genetics of somatic mammalian cells: Genetic, immunologic, and biochemical analysis with Chinese hamster cell hybrids containing selected human chromosomes. Proc. Natl. Acad. Sci. USA, 60, 1275-1281.

Karlseder, J.,Zeillinger, R.,Schneeberger, C.,Czerwenka, K.,Speiser, P.,Kubista, E.,Birnbaum, D.,Gaudray, P., and Theillet, C. (1994). Patterns of DNA amplification at band-q13 of chromosome-11 in human breast-cancer. Genes Chromosomes Cancer, 9, 42-48.

Kato, I.,Tominaga, S., and Terao, C. (1989). Alcohol consumption and cancers of hormone-related organs in females. Jpn J Clin Oncol, 19, 202-7.

Kaufman, D. W.,Kelly, J. P.,Welch, W. R.,Rosenberg, L.,Stolley, P. D.,Warshauer, M. E.,Lewis, J.,Woodruff, J., and Shapiro, S. (1989). Noncontraceptive estrogen use and epithelial ovarian cancer. Am J Epidemiol, 130, 1142-51.

Kaufmann, M.,Vonminckwitz, G.,Kuhn, W.,Schmid, H.,Costa, S.,Goerttler, K., and Bastert, G. (1995). Combination of new biologic parameters as a prognostic index in epithelial ovarian-carcinoma. Int J Gynecol Cancer, 5, 49-55.

Kaya, M.,Yoshida, K.,Higashino, F.,Mitaka, T.,Ishii, S., and Fujinaga, K. (1996). A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene, 12, 221-227.

Khokha, R., andDenhardt, D. T. (1989). Matrix metalloproteinases and tissue inhibitor of metalloproteinases: a review of their role in tumorigenesis and tissue invasion. Invasion Metastasis, 9, 391-405.

Kieback, D. G.,McCamant, S. K.,Press, M. F.,Atkinson, E. N.,Gallager, H. S.,Edwards, C. L.,Hajek, R. A., and Jones, L. A. (1993a). Improved prediction of survival in advanced adenocarcinoma of the ovary by immunocytochemical analysis and the composition adjusted receptor level of the estrogen receptor. Cancer Res, 53, 5188-5192.

Kieback, D. G.,Press, M. F.,Atkinson, E. N.,Edwards, G. L.,Mobus, V. J.,Runnebaum, I. B.,Kreienberg, R., and Jones, L. A. (1993b). Prognostic significance of estrogen receptor expression in ovarian cancer. Immunoreactive Score (IRS) vs. Composition Adjusted Receptor Level (CARL). Anticancer Res, 13, 2489-2496.

Kiechle-Schwarz, M.,Bauknecht, T.,Wienker, T.,Walz, L., and Pfleiderer, A. (1993). Loss of constitutional heterozygosity on chromosome 11p in human ovarian cancer: Positive correlation with grade of differentiation. Cancer, 72, 2423-2432.

Kikuchi, Y.,Kita, T.,Hirata, J., and Fukushima, M. (1994). Preclinical studies of antitumor prostaglandins by using human ovarian-cancer cells. Cancer Metastasis Rev, 13, 309-315.

Kim, W. H.,Jun, S. H.,Kibbey, M. C.,Thompson, E. W., and Kleinman, H. K. (1994). Expression of beta 1 integrin in laminin-adhesion-selected human colon cancer cell lines of varying tumorigenicity. Invasion Metastasis, 14, 147-155.

Klein, K. G., andBouck, N. P. (1994). The distal region of the long arm of human chromosome 1 carries tumor suppressor activity for a human fibrosarcoma line. Cancer Genet Cytogenet, 73, 109-121.

Kleinman, H. K.,McGarvey, M. L.,Liotta, L. A.,Robey, P. G.,Tryggvason, K., and Martin, G. R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry, 21, 6188-6193.

Klemi, P. J.,Takahashi, S.,Joensuu, H.,Kiilholma, P.,Narimatsu, E., and Mori, M. (1994). Immunohistochemical detection of p53 protein in borderline and malignant serous ovarian-tumors. Int J Gynecol Pathol, 13, 228-233.

Klobutcher, L. A., andRuddle, F. H. (1979). Phenotype stabilisation and integration of transferred material in chromosome-mediated gene transfer. Nature, 280, 657-660.

Knudson, A. G. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc.Natl.Acad.Sci.USA, 68, 820-823.

Kohler, M. F.,Marks, J. R.,Wiseman, R. W.,Jacobs, I. J.,Davidoff, A. M.,Clarke-Pearson, D. L.,Soper, J. T.,Bast, R., Jr., and Berchuck, A. (1993). Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. J Natl Cancer Inst, 85, 1513-1519.

Kohler, M. F.,Kerns, B. J. M.,Humphrey, P. A.,Marks, J. R.,Bast, R. C., and Berchuck, A. (1993). Mutation and overexpression of p53 in early-stage epithelial ovarian-cancer. Obstet Gynecol, 81, 643-650.

Koi, M.,Shimizu, M.,Morita, H.,Yamada, H., and Oshimura, M. (1989a). Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion. Jpn. J. Cancer Res., 80, 413-418.

Koi, M.,Morita, H.,Yamada, H.,Satoh, H.,Barrett, J. C., and Oshimura, M. (1989b). Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. Mol Carcinog, 2, 12-21.

Koi, M.,Johnson, L. A.,Kalikin, L. M.,Little, P. F. R.,Nakamura, Y., and Feinberg, A. P. (1993). Tumor-cell growth arrest caused by subchromosomal transferable dna fragments from chromosome-11. Science, 260, 361-364.

Koi, M.,Umar, A.,Chauhan, D. P.,Cherian, S. P.,Carethers, J. M.,Kunkel, T. A., and Boland, C. R. (1994). Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res, 54, 4308-4312.

Kolodner, R. D.,Hall, N. R.,Lipford, J.,Kane, M. F.,Morrison, P. T.,Finan, P. J.,Burn, J.,Chapman, P.,Earabino, C.,Merchant, E., and et, a. (1995). Structure of the human MLH1 locus and analysis of a large hereditary nonpolyposis colorectal carcinoma kindred for mlh1 mutations. Cancer Res, 55, 242-248.

Koufos, A.,Hansen, M. F.,Copeland, N. G.,Jenkins, N. A.,Lampkin, B. C., and Cavenee, W. K. (1985). Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. Nature, 316, 330-334.

Kugoh, H. M.,Hashiba, H.,Shimizu, M., and Oshimura, M. (1990). Suggestive evidence for functionally distinct, tumor-suppressor genes on chromosomes 1 and 11 for a human fibrosarcoma cell line, HT1080. Oncogene, 5, 1637-1644.

Kupryjanczyk, J.,Thor, A. D.,Beauchamp, R.,Merritt, V.,Edgerton, S. M.,Bell, D. A., and Yandell, D. W. (1993). P53 gene-mutations and protein accumulation in human ovarian-cancer. Proc Natl Acad Sci U S A, 90, 4961-4965.

Kurman, R. J., andTrimble, C. L. (1993). The behavior of serous tumors of low malignant potential: Are they ever malignant? Int J Gynecol Pathol, 12, 120-127.

Lapidus, R.G., Ferguson, A.T., Ottaviano, Y.L., Fritz, F.F., Smith, H.S., Weitzman, S.A., Baylin, S.B., Issa, J-P.J., and Davidson, N.E. (1996). Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clinical Cancer Res, 2, 805-810.

Langdon, S. P.,Hirst, G. L.,Miller, E. P.,Hawkins, R. A.,Tesdale, A. L.,Smyth, J. F., and Miller, W. R. (1994). The regulation of growth and protein expression by estrogen in vitro: a study of 8 human ovarian carcinoma cell lines. J Steroid Biochem Molec Biol, 50, 131-155.

Langman, J. (1975). Urogenital System. In Medical Embryology (p. 160). Baltimore: Williams & Wilkins.

Languino, L. R.,Gehlsen, K. R.,Wayner, E.,Carter, W. G.,Engvall, E., and Ruoslahti, E. (1989). Endothelial cells use alpha 2 beta 1 integrin as a laminin receptor. J Cell Biol, 109, 2455-2462.

Larsson, C.,Skogseid, B.,Obberg, K.,Nakamura, Y., and Nordenskjold, N. (1988). Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in inslinoma. Nature, 332, 85-87.

Leach, F. S.,Tokino, T.,Meltzer, P.,Burrell, M.,Oliner, J. D.,Smith, S.,Hill, D. E.,Sidransky, D.,Kinzler, K. W., and Vogelstein, B. (1993). p53 mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res, 53, 2231.

Lee, J. H.,Kavanagh, J. J.,Wharton, J. T.,Wildrick, D. M., and Blick, M. (1989). Allele loss at the c-Ha-ras1 locus in human ovarian cancer. Cancer Res, 49, 1220-1222.

Lee, J. H.,Kavanagh, J. J.,Wildrich, D. M.,Wharton, J. T., and Blick, M. (1990). Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas. Cancer Res, 50, 2724-2728.

Lee, E. C.,Lotz, M. M.,Steele, G., Jr., and Mercurio, A. M. (1992). The integrin alpha 6 beta 4 is a laminin receptor. J Cell Biol, 117, 671-678.

Lee, M. H.,Reynisdottir, I., and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev, 9, 639-649.

Levesque, M. A.,Katsaros, D.,Yu, H.,Zola, P.,Sismondi, P.,Giardina, G., and Diamandis, E. P. (1995). Mutant p53 protein overexpression is associated with poor outcome in patients with well or moderately differentiated ovarian-carcinoma. Cancer, 75, 1327-1338.

Li, S. B.,Schwartz, P. E.,Lee, W. H., and Yangfeng, T. L. (1991). Allele loss at the retinoblastoma locus in human ovarian-cancer. Journal of The National Cancer Institute, 83, 637-640.

Liebman, J. M.,Burbelo, P. D.,Yamada, Y.,Fridman, R., and Kleinman, H. K. (1993). Altered expression of basement-membrane components and collagenases in ascitic xenografts of OVCAR-3 ovarian cancer cells. Int J Cancer, 55, 102-109.

Liotta, L.A.,Lee, W.C., and Morakis, D.J. (1980). New method for preparing large surface of intact basement membrane for tumour invasion studies. Cancer Lett., 11, 141-147.

Liotta, L. A.,Goldfarb, R. H.,Brundage, R.,Siegal, G. P.,Terranova, V., and Garbisa, S. (1981). Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. Cancer Res, 41, 4629-4636.

Liotta, L. A.,Rao, N. C.,Barsky, S. H., and Bryant, G. (1984). The laminin receptor and basement membrane dissolution: role in tumour metastasis. Ciba Found Symp, 108, 146-162.

Lisitsyn, N.,Lisitsyn, N., and Wigler, M. (1993). Cloning the differences between two complex genomes. Science, 259, 946-951.
Litt, M.,Sharma, V., and Luty, J. (1990). Dinucleotide repeat polymorphism at the D11S35 locus. Nucleic Acids Res, 18, 5921.

Litt, M.,Kramer, P.,Hauge, X. Y.,Weber, J. L.,Wang, Z.,Wilkie, P. J.,Holt, M. S.,Mishra, S.,Doniskeller, H., and Warnich, L. (1993). A microsatellite-based index map of human chromosome-11. Hum Mol Genet, 2, 909-913.

Littlefield, J. (1964). Selection of hybrids from mating of fibroblasts in-vitro and their presumed recombinants. Science, 145, 709-710.

Loh, W., Jr.,Scrable, H. J.,Livanos, E.,Arboleda, M. J.,Cavenee, W. K.,Oshimura, M., and Weissman, B. E. (1992). Human chromosome 11 contains two different growth suppressor genes for embryonal rhabdomyosarcoma. Proc Natl Acad Sci U S A, 89, 1755-1759.

Lothe, R. A.,Fossa, S. D.,Stenwig, A. E.,Nakamura, Y.,White, R.,Borresen, A. L., and Brogger, A. (1989). Loss of 3p or 11p alleles is associated with testicular cancer tumors. Genomics, 5, 134-138.

Lugo, T. G.,Handelin, B.,Killary, A. M.,Housman, D. E., and Fournier, R. E. (1987). Isolation of microcell hybrid clones containing retroviral vector insertions into specific human chromosomes. Mol Cell Biol, 7, 2814-2820.

Lupton, S.D.,Brunton, L.L.,Kalberg, V.A., and Overell, K.W. (1991). Dominant positive and negative selection using a hygromycin phosphotranferase-thymidine kinase fusion gene. Mol Cell Biol, 11, 3374-3378.

Lynch, H. T.,Kimberling, W.,Albano, W. A.,Lynch, J. F.,Biscone, K.,Schuelke, G. S.,Sandberg, A. A.,Lipkin, M.,Deschner, E. E.,Mikol, Y. B., (1985a). Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). I. Clinical description of resource. Cancer, 56, 934-938.

Lynch, H. T.,Schuelke, G. S.,Kimberling, W. J.,Albano, W. A.,Lynch, J. F.,Biscone, K. A.,Lipkin, M. L.,Deschner, E. E.,Mikol, Y. B.,Sandberg, A. A., (1985b). Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). II. Biomarker studies. Cancer, 56, 939-91.

Lynch, H. T., and Lynch, J. F. (1993). The Lynch syndromes. Curr Opin Oncol, 5, 687-696.

Maemura, M.,Akiyama, S. K.,Woods, V., Jr., and Dickson, R. B. (1995). Expression and ligand binding of alpha 2 beta 1 integrin on breast carcinoma cells. Clin Exp Metastasis, 13, 223-235.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989). Molecular Cloning: A laboratory manual, 2nd ed. Cold Spring Harbor publications, USA.

Marcel, M.M.,DeBruyere, G.K.,Vandesande, F., and Dragonetti, C. (1981). Immunohistochemical study of embryonic chick heart invaded by malignant cells in three dimensional culture. Invasion Metastasis, 1, 195-204.

Markman, M.,Berek, J. S.,Blessing, J. A.,McGuire, W. P.,Bell, J., and Homesley, H. D. (1992). Characteristics of patients with small-volume residual ovarian cancer unresponsive to cisplatin-based ip chemotherapy: Lessons learned from a Gynecologic Oncology Group phase II trial of ip cisplatin and recombinant alpha- interferon. Gynecol Oncol, 45, 3-8.

Marquis, S. T.,Rajan, J. V.,Wynshawboris, A.,Xu, T. J.,Yin, G. Y.,Abel, K. J.,Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of brca1 expression implies a role in differentiation of the breast and other tissues. Nature Genet, 11, 17-26.

Marx, J. (1994). How cells cycle towards cancer. Science, 263, 319.
Masood, S.,Heitmann, J.,Nuss, R. C., and Benrubi, G. I. (1989). Clinical correlation of hormone receptor status in epithelial ovarian cancer. Gynecol Oncol, 34, 57-60.

Matsuoka, S.,Edwards, M. C.,Bai, C.,Parker, S.,Zhang, P.,Baldini, A.,Harper, J. W., and Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev, 9, 650-662.

McBride, O. W., andOzer, H. L. (1973). Transfer of genetic information by purified metaphase chromosomes. Proc Natl Acad Sci U S A, 70, 1258-1262.

McClay, E. F.,Albright, K. D.,Jones, J. A.,Eastman, A.,Christen, R., and Howell, S. B. (1992). Modulation of cisplatin resistance in human malignant melanoma cells. Cancer Res, 52, 6790-6796.

McGill, J. R.,Beitzel, B. F.,Nielsen, J. L.,Walsh, J. T.,Drabek, S. M.,Meader, R. J., and Vonhoff, D. D. (1993). Double minutes are frequently found in ovarian carcinomas. Cancer Genet Cytogenet, 71, 125-131.

McGill, J.,Mattern, V.,Beitzel, B.,Leach, R.,Morrow, M.,Hodge, J.,Johnson-Pais, T.,Von-Hoff, D., and Eckhardt, S. (1996). Microdissected double minute DNA facilitates discovery of genomic alterations in human ovarian cancer (abstract 871). Proc Am Assoc Cancer Res, 37, 126.

McGowan-Jordan, I. J.,Speevak, M. D.,Blakey, D., and Chevrette, M. (1994). Suppression of tumorigenicity in human teratocarcinoma cell line PA-1 by introduction of chromosome 4. Cancer Res, 54, 2568-2572.

McGuire, W. P.,Hoskins, W. J.,Brady, M. F.,Kucera, P. R.,Partridge, E. E.,Look, K. Y., and Davidson, M. (1995). Taxol and cisplatin (TP) improves outcome in advanced ovarian cancer (AOC) as compared to cytoxan and cispatin (CP) (meeting abstract). In Proc Annu Meet Am Soc Clin Oncol, 14 (pp. 275).

McNeill, C. A., andBrown, R. L. (1980). Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc Natl Acad Sci U S A, 77, 5394-5398.

Merajver, S. D.,Pham, T. M.,Caduff, R. F.,Chen, M.,Poy, E. L.,Cooney, K. A.,Weber, B. L.,Collins, F. S.,Johnston, C., and Frank, T. S. (1995). Somatic mutations in the BRCA1 gene in sporadi ovarian tumours. Nature Genet., 9, 439-443.

Miele, M. E.,Robertson, G.,Lee, J. H.,Coleman, A.,McGary, C. T.,Fisher, P. B.,Lugo, T. G., and Welch, D. R. (1996). Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human-melanoma cell-line meljuso after introduction of human-chromosome-1 or human-chromosome-6. Mol Carcinog, 15, 284-299.

Migeon, B. R., andMiller, C. S. (1968). Human-Mouse somatic cell hybrids with single human chromosome (Group E): Link with thymidine kinase activity. Science, 162, 1005-1006.

Miki, Y.,Swensen, J.,Shattuck-Eidens, D.,Futreal, P. A.,Harshman, K.,Tavtigian, S.,Liu, Q.,Cochran, C.,Bennett, L. M.,Ding, W., and et, a. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science, 266, 66-71.

Milner, B. J.,Miler, I. D.,Kitchener, H. C.,Parkin, D., and Haites, N. E. (1996). The p21-WAF1 gene is infrequently mutated in human ovarian tumours and p21-WAF1 protein levels appear to be regulated independently of p53 status (abstract 3562). Proc Am Assoc Cancer Res, 37 (pp. 521). Washington.

Misra, B. C., andSrivatsan, E. S. (1989). Localization of HeLa cell tumor-suppressor gene to the long arm of chromosome II. Am J Hum Genet, 45, 565-577.

Mittal, K. R.,Zeleniuch-Jacquotte, A.,Cooper, J. L., and Demopoulos, R. I. (1993). Contralateral ovary in unilateral ovarian carcinoma: A search for preneoplastic lesions. Int J Gynecol Pathol, 12, 59-63.

Morgan, D. O. (1995). Principles of CDK regulation. Nature, 374, 131.

Mousavi, Y., andAdlercreutz, H. (1993). Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. Steroids, 58, 301-4.

Nakata, B.,Albright, K. D.,Barton, R. M.,Howell, S. B., and Los, G. (1995). Synergistic interaction between cisplatin and tamoxifen delays the emergence of cisplatin resistance in head and neck cancer cell lines. Cancer Chemother Pharmacol, 35, 511-518.

Narod, S. A. (1994). Genetics of breast and ovarian cancer. Br Med Bull, 50, 656-676.

Negrini, M.,Sabbioni, S.,Possati, L.,Rattan, S.,Corallini, A.,Barbanti-Brodano, G., and Croce, C. M. (1994). Suppression of tumorigenicity of breast cancer cells by microcell-mediated chromosome transfer: studies on chromosomes 6 and 11. Cancer Res, 54, 1331-1336.

Negrini, M.,Rasio, D.,Hampton, G. M.,Sabbioni, S.,Rattan, S.,Carter, S. L.,Rosenberg, A. L.,Schwartz, G. F.,Shiloh, Y.,Cavenee, W. K., and Croce, C. M. (1995). Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. Cancer Res, 55, 3003-3007.

Nehme, A.,Julia, A. M.,Jozan, S.,Chevreau, C.,Bugat, R., and Canal, P. (1994). Modulation of cisplatin cytotoxicity by human recombinant interferon-gamma in human ovarian cancer cell lines. Eur J Cancer. 30a, 4, 520-525.

Neijt, J. P. (1994). Advances in the chemotherapy of gynecologic cancer. Curr Opin Oncol, 6, 531-538.

Neuhausen, S.,Gilewski, T.,Norton, L.,Tran, T.,McGuire, P.,Swensen, J.,Hampel, H.,Borgen, P.,Brown, K.,Skolnick, M.,Shattuckeidens, D.,Jhanwar, S.,Goldgar, D., and Offit, K. (1996). Recurrent brca2 6174delt mutations in ashkenazi jewish women affected by breast-cancer. Nature Genet, 13, 126-128.

Nicolaides, N. C.,Papadopoulos, N.,Liu, B.,Wei, Y. F.,Carter, K. C.,Ruben, S. M.,Rosen, C. A.,Haseltine, W. A.,Fleischmann, R. D.,Fraser, C. M., and et, a. (1994). Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature, 371, 75-80.

Nigg, E. A. (1995). Cycin dependent protein kinases: Key regulators of the eukaryotic cell cycle. Bioessays, 17, 471.

Niwa, K.,Itoh, M.,Murase, T.,Morishita, S.,Itoh, N.,Mori, H., and Tamaya, T. (1994). Alteration of p53 gene in ovarian-carcinoma - clinicopathological correlation and prognostic-significance. Br J Cancer, 70, 1191-1197.

Nobori, T.,Miura, K.,Wu, D. J.,Lois, A.,Takabayashi, K., and Carson, D. A. (1994). Deletions of the cyclin dependent kinase 4 inhibitor gene in multiple human cancers. Nature, 368, 753.

O'Brien, M. E.,Schofield, J. B.,Tan, S.,Fryatt, I.,Fisher, C., and Wiltshaw, E. (1993). Clear cell epithelial ovarian cancer (mesonephroid): Bad prognosis only in early stages. Gynecol Oncol, 49, 250-254.

O'Malley, B. W. (1990). The steroid receptor superfamily: More excitement predicted for the future. Mol Endocrinol, 4, 363-369.

Ohta, M.,Inoue, H.,Cotticelli, M. G.,Kastury, K.,Baffa, R.,Palazzo, J.,Siprashvili, Z.,Mori, M.,McCue, P.,Druck, T.,Croce, C. M., and Huebner, K. (1996). The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell, 84, 587-97.

Olt, G.,Miller, S., and Satyaswaroop, P. G. (1996). Laminin abolishes adhesion of ovarian cancer cells to peritoneum and malignant progression, in vivo (abstract 462). Proc Am Assoc Cancer Res, 37, 66.

Orkin, S. H.,Goldman, D. S., and Sallan, S. E. (1984). Development of homozygosity for chromosome 11p markers in Wilms' tumour. Nature, 309, 172-174.

Orlow, I.,Iavarone, A.,Crider-Miller, S. J.,Bonilla, F.,Latres, E.,Lee, M. H.,Gerald, W. L.,Massague, J.,Weissman, B. E., and Cordon-Cardo, C. (1996). Cyclin-dependent kinase inhibitor p57KIP2 in soft tissue sarcomas and Wilms'tumors. Cancer Res, 56, 1219-1221.

Orphanos, V.,McGown, G.,Hey, Y.,Thorncroft, M.,Santibanez-Koref, M.,Russell-SEH,Hickey, I.,Atkinson, R. J., and Boyle, J. M. (1995). Allelic imbalance of chromosome 6q in ovarian tumours. BJC, 71, 666-669.

Osborne, R. J., and Leech, V. (1994). Polymerase chain-reaction allelotyping of human ovarian-cancer. Br J Cancer, 69, 429-438.

Oshimura, M.,Kugoh, H.,Koi, M.,Shimizu, M.,Yamada, H.,Satoh, H., and Barrett, J. C. (1990). Transfer of a normal human chromosome 11 suppresses tumorigenicity of some but not all tumor cell lines. J Cell Biochem, 42, 135-142.

Parazzini, F.,Franceschi, S.,La-Vecchia, C., and Fasoli, M. (1991). The epidemiology of ovarian cancer. Gynecol Oncol, 43, 9-23.

Park, J. S.,Kim, H. K.,Han, S. K.,Lee, J. M.,Namkoong, S. E., and Kim, S. J. (1995). Detection of c-k-ras point mutation in ovarian-cancer. Int J Gynecol Cancer, 5, 107-111.

Pejovic, T.,Heim, S.,Mandhal, N.,Baldetorp, B.,Elmfors, B.,Floderus, U.-M.,Furgyik, S.,Helm, G.,Himmelman, A.,Willen, H., and Mitelman, F. (1992). chromosome aberration in 35 primary ovarian carcinomas. Genes Chromosom. Cancer, 4, 58-68.

Pellegrini, R.,Martignone, S.,Tagliabue, E.,Belotti, D.,Bufalino, R.,Cascinelli, N.,Menard, S., and Colnaghi, M. I. (1995). Prognostic significance of laminin production in relation with its receptor expression in human breast carcinomas. Breast Cancer Res Treat, 35, 195-199.

Pershouse, M. A.,Stubblefield, E.,Hadi, A.,Killary, A. M.,Yung, W. K., and Steck, P. A. (1993). Analysis of the functional role of chromosome 10 loss in human glioblastomas. Cancer Res, 53, 5043-5050.

Phelan, C. M.,Rebbeck, T. R.,Weber, B. L.,Devilee, P.,Ruttledge, M. H.,Lynch, H. T.,Lenoir, G. M.,Stratton, M. R.,Easton, D. F.,Ponder, B. A. J.,Cannonalbright, L.,Larsson, C.,Goldgar, D. E., and Narod, S. A. (1996a). Ovarian-cancer risk in brca1 carriers is modified by the hras1 variable number of tandem repeat (vntr) locus. Nature Genet, 12, 309-311.

Phelan, C. M.,Lancaster, J. M.,Tonin, P.,Gumbs, C.,Cochran, C.,Carter, R.,Ghadirian, P.,Perret, C.,Moslehi, R.,Dion, F.,Faucher, M. C.,Dole, K.,Karimi, S.,Foulkes, W.,Lounis, H.,Warner, E.,Goss, P.,Anderson, D.,Larsson, C.,Narod, S. A., and Futreal, P. A. (1996b). Mutation analysis of the brca2 gene in 49 site-specific breast-cancer families. Nature Genet, 13, 120-122.

Phillips, K. K.,Arnold, J. T., and Weissman, B. E. (1994). Effects on the in-vitro and in-vivo growth-properties of a human breast-carcinoma cell-line and wilms-tumor cell-line following microcell-mediated transfer of a normal human-chromosome-16. J Cell Biochem, 196-196.

Phillips, K. K.,Welch, D. R.,Miele, M. E.,Lee, J. H.,Wei, L. L., and Weissman, B. E. (1996). Suppression of mda-mb-435 breast-carcinoma cell metastasis following the introduction of human-chromosome-11. Cancer Res, 56, 1222-1227.

Puls, L. E.,Powell, D. E.,DePriest, P. D.,Gallion, H. H.,Hunter, J. E.,Kryscio, R. J., and van, N. J., Jr. (1992). Transition from benign to malignant epithelium in mucinous and serous ovarian cystadenocarcinoma. Gynecol Oncol, 47, 53-57.

Rasio, D.,Negrini, M.,Maneneti, G.,Draggani, T. A., and Croce, C. M. (1995). Loss of heterozygosity at chromosome 11q in lung adenocarcinoma: identification of three independent regions. Cancer Res, 55, 3988-3991.

Rasio, D.,Negrini, M., and Croce, C. M. (1995). Genomic organization of the atm locus involved in ataxia-telangiectasia. Cancer Res, 55, 6053-6057.

Reeve, A. E.,Housiaux, P. J.,Gardner, R. J.,Chewings, W. E.,Grindley, R. M., and Millow, L. J. (1984). Loss of a Harvey ras allele in sporadic Wilms' tumour. Nature, 309, 174-6.

Reeve, A. E.,Sih, S. A.,Raizis, A. M., and Feinberg, A. P. (1989). Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumour cells. Mol. Cell. Biol., 9, 1799-1803.

Reid, L. H.,Crider-Miller, S. J.,West, A.,Lee, M. H.,Massague, J., and Weissman, B. E. (1996). Genomic organization of the human p57KIP2 gene and its analysis in the G401 Wilms' tumor assay. Cancer Res, 56, 1214-1218.

Rendina, G. M.,Donadio, C., and Giovanni, M. (1982). Steroid receptors and progestinic therapy in ovarian endometrioid carcinoma. Eur J Gynaec Oncol, 3, 241-245.

Rimessi, P.,Gualandi, F.,Morelli, C.,Trabanelli, C.,Wu, Q.,Possati, L.,Montesi, M.,Barrett, J. C., and Barbanti-Brodano, G. (1994). Transfer of human chromosome 3 to an ovarian carcinoma cell line identifies three regions on 3p involved in ovarian cancer. Oncogene, 9, 3467-3474.

Rinker-Schaeffer, C. W.,Hawkins, A. L.,Ru, N.,Dong, J.,Stoica, G.,Griffin, C. A.,Ichikawa, T.,Barrett, J. C., and Isaacs, J. T. (1994). Differential suppression of mammary and prostate cancer metastasis by human chromosomes 17 and 11. Cancer Res, 54, 6249-6256.

Risch, H. A.,Jain, M.,Marrett, L. D., and Howe, G. R. (1994). Dietary fat intake and risk of epithelial ovarian cancer. J Natl Cancer Inst, 86, 1409-1415.

Rodriguez-Alfageme, C.,Stanbridge, E. J., and Astrin, S. M. (1992). Suppression of deregulated c-MYC expression in human colon carcinoma cells by chromosome 5 transfer. Proc Natl Acad Sci U S A, 89, 1482-1486.

Romagnolo, D.,Annab, L. A.,Risinger, J. I.,Terry, L. A.,Lyon, T. T., and Barrett, J. C. (1996). Regulation of expression of BRCA1 by estrogenin breast MCF-7 and ovarian BG-1 cancer cells (Abstract 3532). Proc Am Assoc Cancer Res, 37 (pp. 516). Washington.

Ron, E.,Lunenfeld, B.,Menczer, J.,Blumstein, T.,Katz, L.,Oelsner, G., and Serr, D. (1987). Cancer incidence in a cohort of infertile women. Am J Epidemiol, 125, 780-790.

Rose, P. G.,Reale, F. R.,Longcope, C., and Hunter, R. E. (1990). Prognostic significance of estrogen and progesterone receptors in epithelial ovarian cancer. Obstet Gynecol, 76, 258-263.

Rose, D. P.,Goldman, M.,Connolly, J. M., and Strong, L. E. (1991). High-fiber diet reduces serum estrogen concentrations in premenopausal women. Am J Clin Nutr, 54, 520-525.

Rosen, A.,Sevelda, P.,Klein, M.,Dobianer, K.,Hruza, C.,Czerwenka, K.,Hanak, H.,Vavra, N.,Salzer, H., and Leodolter, S. (1993). 1st experience with FGF-3 (int-2) amplification in women with epithelial ovarian-cancer. Br J Cancer, 67, 1122-1125.

Rossing, M. A.,Daling, J. R.,Weiss, N. S.,Moore, D. E., and Self, S. G. (1994). Ovarian tumors in a cohort of infertile women. N Engl J Med, 331, 771-776.

Rousseau-Merck, M. F.,Misrahi, M.,Loosfelt, H.,Milgrom, E., and Berger, R. (1987). Localisation of the human progesterone receptor gene to chromosome 11q22. Hum Genet, 77, 280-282.

Rowe, S. M.,Coughlan, S. J.,McKenna, N. J.,Garrett, E.,Kieback, D. G.,Carney, D. N., and Headon, D. R. (1995). Ovarian carcinoma-associated TaqI restriction fragment length polymorphism in intron G of the progesterone receptor gene is due to an Alu sequence insertion. Cancer Res, 55, 2743-2745.

Sanchez, Y.,Lovell, M.,Marin, M. C.,Wong, P. E.,Wolfledbetter, M. E.,McDonnell, T. J., and Killary, A. M. (1996). Tumor suppression and apoptosis of human prostate carcinoma mediated by a genetic-locus within human-chromosome 10pter-q11. Proc Natl Acad Sci U S A, 93, 2551-2556.

Sandhu, A. K.,Hubbard, K.,Kaur, G. P.,Jha, K. K.,Ozer, H. L., and Athwal, R. S. (1994). Senescence of immortal human fibroblasts by the introduction of normal human-chromosome-6. Proc Natl Acad Sci U S A, 91, 5498-5502.

Sandhu, A. K.,Kaur, G. P.,Reddy, D. E.,Rane, N. S., and Athwal, R. S. (1996). A gene on 6q14-21 restores senescence to immortal ovarian tumor-cells. Oncogene, 12, 247-252.

Sanford, J. A., andStubblefield, E. (1987). General protocol for microcell-mediated chromosome transfer. Somat Cell Mol Genet, 13, 279-284.

Sato, T.,Saito, H.,Morita, R.,Koi, S.,Lee, J. H., and Nakamura, Y. (1991). Allelotype of human ovarian cancer. Cancer Res., 51, 5118-5122.

Satoh, H.,Lamb, P. W.,Dong, J. T.,Everitt, J.,Boreiko, C.,Oshimura, M., and Barrett, J. C. (1993). Suppression of tumorigenicity of A549 lung adenocarcinoma cells by human chromosomes 3 and 11 introduced via microcell-mediated chromosome transfer. Mol Carcinog, 7, 157-164.

Savitsky, K.,Barshira, A.,Gilad, S.,Rotman, G.,Ziv, Y.,Vanagaite, L.,Tagle, D. A.,Smith, S.,Uziel, T., and Sfez, S. (1995). A single ataxia-telangiectasia gene with a product similar to pi-3 kinase. Science, 268, 1749-1753.

Saxon, P. J.,Srivatsan, E. S.,Leipzig, G. V.,Sameshima, J. H., and Stanbridge, E. J. (1985). Selective transfer of individual human chromosomes to recipient cells. Mol Cell Biol, 5, 140-146.

Saxon, P. J.,Srivatsan, E. S., and Stanbridge, E. J. (1986). Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. Embo J, 5, 3461-3466.

Saxon, P. J., andStanbridge, E. J. (1987). Transfer and selective retention of single specific human chromosomes via microcell-mediated chromosome transfer. Methods Enzymol, 151, 313-325.

Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., Van Heyningen, V., Hastie, N. D.(1996). Influence of pax6 gene dosage on development - overexpression causes severe eye abnormalities. Cell, 86, 71-82

Schlechte, W.,Brattain, M., and Boyd, D. (1990). Invasion of the extra-cellular matrix by cultured colon cancer cells: dependence on urokinase receptor display. Cancer Commun., 2, 173-179.

Schultz, D. C.,Vanderveer, L.,Buetow, K. H.,Boente, M. P.,Ozols, R. F.,Hamilton, T. C., and Godwin, A. K. (1995). Characterisation of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including cdkn2. Cancer Res, 55, 2150.

Schwartz, P. E.,Chambers, J. T.,Kohorn, E. I.,Chambers, S. K.,Weitzman, H.,Voynick, I. M.,MacLusky, N., and Naftolin, F. (1989). Tamoxifen in combination with cytotoxic chemotherapy in advanced epithelial ovarian cancer. A prospective randomized trial. Cancer, 63, 1074-1078.

Scrable, H. J.,Witte, D. P.,Lampkin, B. C., and Cavenee, W. K. (1987). Chromosomal localization of the human rhabdomyosarcoma locus by mitotic recombination mapping. Nature, 329, 645-647.

Sevelda, P.,Schemper, M., and Spona, J. (1989). CA 125 as an independent prognostic factor for survival in patients with epithelial ovarian cancer. Am J Obstet Gynecol, 161, 1213-1216.

Sevelda, P.,Denison, U.,Schemper, M.,Spona, J.,Vavra, N., and Salzer, H. (1990). Oestrogen and progesterone receptor content as a prognostic factor in advanced epithelial ovarian carcinoma. Br J Obstet Gynaecol, 97, 706-712.

Shelling, A. N.,Cooke, I. E., and Ganesan, T. S. (1995). The genetic-analysis of ovarian-cancer. Br J Cancer, 72, 521-527.

Sheridan, E.,Silcocks, P.,Smith, J.,Hancock, B. W., and Goyns, M. H. (1994). P53 mutation in a series of epithelial ovarian cancers from the uk, and its prognostic-significance. Eur J Cancer, 11, 1701-1704.

Shimizu, M.,Yokota, J.,Mori, N.,Shuin, T.,Shinoda, M.,Terada, M., and Oshimura, M. (1990). Introduction of normal chromosome 3p modulates the tumorigenicity of a human renal cell carcinoma cell line YCR. Oncogene, 5, 185-194.

Slamon, D. J.,Godolphin, W.,Jones, L. A.,Holt, J. A.,Wong, S. G.,Keith, D. E.,Levin, W. J.,Stuart, S. G.,Udove, J., and Ullrich, A. (1989). Studies of the Her-2 / neu proto-oncogene in human breast and ovarian cancer. Science, 244, 707.

Slotman, B. J., andRao, B. R. (1988). Ovarian Cancer (Review). Anticancer Research, 8, 417-434.

Smyth, J.,Bowman, A.,Perren, T.,Wilkinson, P.,Prescott, R.,Quinn, K., and Tedeschi, M. (1995). Glutathione improves the therapeutic index of cisplatin and quality of life for patients with ovarian cancer (meeting abstract). In Proc Annu Meet Am Soc Clin Oncol, 14 (pp. 273).

Southern, E. M. (1975). Detection of specific sequences amongst DNA fragments separated by agarose gel electrophoresis. J. Mol. Biol., 98, 503-517.

Sozzi, G.,Veronese, M. L.,Negrini, M.,Baffa, R.,Cotticelli, M. G.,Inoue, H.,Tornielli, S.,Pilotti, S.,De-Gregorio, L.,Pastorino, U.,Pierotti, M. A.,Ohta, M.,Huebner, K., and Croce, C. M. (1996). The FHIT gene 3p14.2 is abnormal in lung cancer. Cell, 85, 17-26.

Spirtas, R.,Kaufman, S. C., and Alexander, N. J. (1993). Fertility drugs and ovarian cancer: red alert or red herring?. Fertil Steril, 59, 291-293.

Srivatsan, E. S.,Benedict, W. F., and Stanbridge, E. J. (1986). Implication of chromosome 11 in the suppression of neoplastic expression in human cell hybrids. Cancer Res, 46, 6174-6179.

Srivatsan, E. S.,Misra, B. C.,Venugopalan, M., and Wilczynski, S. P. (1991a). Loss of heterozygosity on chromosome 11 in cervical carcinoma. Am. J. Hum. Genet., 49, 868-877.

Srivatsan, E. S.,Murali, V., and Seeger, R. C. (1991b). Loss of heterozygosity for alleles on chromosome 11q and 14q in neuroblastoma. Prog. Clin. Biol. Res., 366, 91-98.

Stanbridge, E. J. (1976). Suppression of malignancy in human cells. Nature, 260, 17-20.

Stanbridge, E. J., andCeredig, R. (1981). Growth-regulatory control of human cell hybrids in nude mice. Cancer Res, 41, 573-580.

Starkey, J.R.,Hosick, H.L.,Stanford, D.R., and Liggit, H.D. (1984). Interaction of metstatic tumour cells with bovine lens. Cancer Res, 44, 1585-1594.

Steel, C. M.,Eccles, D. M.,Gruber, L.,Wallace, M.,Lessels, A.,Morsman, J. M.,Gabra, H.,Leonard, R. C. F., and Cohen, B. B. (1993). Allele losses on chromosome 17 in ovarian tumours. In Proc. Helene Harris Memorial Meeting, (in press). Toronto.

Steichen-Gersdorf, E.,Gallion, H. H.,Ford, D.,Girodet, C.,Easton, D. F.,DiCioccio, R. A.,Evans, G.,Ponder, M. A.,Pye, C.,Mazoyer, S., and et, a. (1994). Familial site-specific ovarian cancer is linked to BRCA1 on 17q12-21 [see comments]. Am J Hum Genet, 55, 870-5.

Struewing, J. P.,Abeliovich, D.,Peretz, T.,Avishai, N.,Kaback, M. M.,Collins, F. S., and Brody, L. C. (1995). The carrier frequency of the BRCA1 185delAG mutation is approximately 1 percent in Ashkenazi Jewish individuals. Nature Genet, 11, 198.

Stubblefield, E., andPershouse, M. (1992). Direct formation of microcells from mitotic cells for use in chromosome transfer. Somat Cell Mol Genet, 18, 485-491.

Sumner, A. T.,Evans, H. J., and Buckland, R. A. (1971). A new technique for distinguishing between human chromosomes. Nature, 232, 31-32.

Sugawara, O.,Oshimura, M.,Koi, M.,Annab, L.A., and Barrett, J.C. (1990). Induction of cellular senescence in immortalized cells by human chromosome 1. Science, 247, 707-710.

Takahashi, H.,Behbakht, K.,McGovern, P. E.,Chiu, H. C.,Couch, F. J.,Weber, B. L.,Friedman, L. S.,King, M. C.,Furusato, M., and Livolsi, V. A. (1995). Mutation analysis of the BRCA1 gene in ovarian cancers. Cancer Res, 55, 2998.

Tanaka, K.,Oshimura, M.,Kikuchi, R.,Seki, M.,Hayashi, T., and Miyaki, M. (1991). Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18. Nature, 349, 340-342.

Tanaka, K.,Yanoshita, R.,Konishi, M.,Oshimura, M.,Maeda, Y.,Mori, T., and Miyaki, M. (1993). Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. Oncogene, 8, 2253-2258.

Tanaka, K.,Kikuchiyanoshita, R.,Muraoka, M.,Konishi, M.,Oshimura, M., and Miyaki, M. (1996). Suppression of tumorigenicity and invasiveness of

colon-carcinoma cells by introduction of normal chromosome 8p12-pter. Oncogene, 12, 405-410.

Tavassoli, M.,Ruhrberg, C.,Beaumont, V.,Reynolds, K.,Kirkham, N.,Collins, W. P., and Farzaneh, F. (1993). Whole chromosome-17 loss in ovarian-cancer. Genes Chromosomes Cancer, 8, 195-198.

Templeton, N. S.,Brown, P. D.,Levy, A. T.,Margulies, I. M.,Liotta, L. A., and Stetler-Stevenson, W. G. (1990). Cloning and characterization of human tumor cell interstitial collagenase. Cancer Res, 50, 5431-5437.

Terranova, V. P.,Hujanen, E. S., and Martin, G. R. (1986). Basement membrane and the invasive activity of metastatic tumour cells. J. Natl. Cancer Inst., 77, 311-316.

Theile, M.,Hartmann, S.,Scherthan, H.,Arnold, W.,Deppert, W.,Frege, R.,Glaab, F.,Haensch, W., and Scherneck, S. (1995). Suppression of tumorigenicity of breast-cancer cells by transfer of human-chromosome-17 does not require transferred brca1 and p53 genes. Oncogene, 10, 439-447.

Thomas, K.R.andCapecchi, M.R. (1987). Site directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell, 51, 503-512.

Thompson, M. E.,Jensen, R. A.,Obermiller, P. S.,Page, D. L., and Holt, J. T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during breast cancer progression. Nature Genet, 9, 444.

Tomlinson, I. P. M.,Gammack, A. J.,Stickland, J. E.,Mann, G. J.,MacKie, R. M.,Kefford, R. F., and McGee, J. O. (1993). Loss of heterozygosity in malignant-melanoma at loci on chromosome-11 and chromosome-17 implicated in the pathogenesis of other cancers. Genes Chromosomes Cancer, 7, 169-172.

Tourian, A.,Johnson, R. T.,Burg, K.,Nicolson, S. W., and Sperling, K. (1978). Transfer of human chromosomes via human minisegregant cells into mouse cells and the quantitation of the expression of hypoxanthine phosphoribosyltransferase in the hybrids. J Cell Sci, 30, 193-209.

Toyoshima, H., andHunter, T. (1994). p27, a novel inhibitor of G1 cyclin-CDK protein kinase activity is related to p21. Cell, 78, 67.

Trent, J. M.,Stanbridge, E. J.,McBride, H. L.,Meese, E. U.,Casey, G.,Araujo, D. E.,Witkowski, C. M., and Nagle, R. B. (1990). Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. Science, 247, 568-571.

Tunnacliffe, A.,Jones, C., and Goodfellow, P. (1983). Somatic cell genetics, immunogenetics and gene mapping. Immunol. Today, 4, 230-233.

Uzawa, N.,Yoshida, M. A.,Oshimura, M., and Ikeuchi, T. (1995). Suppression of tumorigenicity in three different cell lines of human oral squamous cell carcinoma by introduction of chromosome 3p via microcell-mediated chromosome transfer. Oncogene, 11, 1997-2004.

van den Brule, F. A.,Taraboletti, G.,Berchuck, A.,Belotti, D.,Menard, S., and Castronovo, V. (1996). Expression of the 67 kD laminin receptor in ovarian and endometrial carcinomas (abstract 467). Proc Am Assoc Cancer Res, 37, 67.

Van Heyningen, V., andLittle, P. F. R. (1995a). Report of the fourth international workshop on human chromosome 11 mapping 1994. Cytogenet Cell Genet, 69, 128-158.

Van Heyningen, V., Schedl, A., Danes, S., Fantes, J. A., Hanson, I. M., Tifft, C., Hastie, N. D. (1995b). Yac-transgenic correction of mutant smalleye phenotype used to explore aniridia associated position effect and gene dosage of the pax6 gene. Am J Hum Genet, 57, 267

Vandamme, B.,Lissens, W.,Amfo, K.,De-Sutter, P.,Bourgain, C.,Vamos, E., and De-Greve, J. (1992). Deletion of chromosome 11p13-11p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. Cancer Res, 52, 6646-6652.

Vanderzee, A. G. J.,Hollema, H.,Suurmeijer, A. J. H.,Krans, M.,Sluiter, W. J.,Willemse, P. H. B.,Aalders, J. G., and Devries, E. G. E. (1995). Value of p-glycoprotein, glutathione-s-transferase-pi, c-erbb-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol, 13, 70-78.

Vermes, I.,Haanen, C.,Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods, 184, 39-51.

Viel, A.,Giannini, F.,Tumiotto, L.,Sopracordevole, F.,Visentin, M. C., and Boiocchi, M. (1992). Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. Br J Cancer, 66, 1030-1036.

Viel, A.,Giannini, F.,Capozzi, E.,Canzonieri, V.,Scarabelli, C.,Gloghini, A., and Boiocchi, M. (1994). Molecular mechanisms possibly affecting wt1 function in human ovarian-tumors. Int J Cancer, 57, 515-521.

Viel, A.,Dallagnese, L.,Canzonieri, V.,Sopracordevole, F.,Capozzi, E.,Carbone, A.,Visentin, M. C., and Boiocchi, M. (1995). Suppressive role of the metastasis-related nm23-h1 gene in human ovarian carcinomas - association of high messenger-RNA expression with lack of lymph-node metastasis. Cancer Res, 55, 2645-2650.

Vogelstein, B., andKinzler, K. W. (1993). The multistep nature of cancer. Trends Genet., 9, 138-141.

Vorechovsky, I.,Rasio, D.,Luo, L. P.,Monaco, C.,Hammarstrom, L.,Webster, A. D. B.,Zaloudik, J.,Barbantibrodano, G.,James, M.,Russo, G.,Croce, C. M., and Negrini, M. (1996). The atm gene and susceptibility to breast-cancer - analysis of 38 breast-tumors reveals no evidence for mutation. Cancer Res, 56, 2726-2732.

Wan, M.,Vyas, R.,Zheng, J., and Dubeau, L. (1996). Introduction of a normal human chromosome 6 into ovarian carcinoma cell lines controls their tumorigenic expression (abstract 4052). In Proc Am Assoc Cancer Res, 37 (pp. 591). Washington.

Wang, S.,Konig, R.,Kohler, T.,Stickeler, E.,Tong, X. W.,Kieback, D. G.,Kreienberg, R., and Runnebaum, I. B. (1996). p53 polymorphism p53PIN3 in ovarian and breast cancer patients (abstract 1785). In Proc A m Assoc Cancer Res, 37 (pp. 262). Washington.

Watson, J. E. V.,Slorach, E. M.,Maule, J.,Lawson, D.,Porteous, D. J., and Brookes, A. J. (1995). Human repeat-mediated integration of selectable markers into somatic-cell hybrids. Genome Res, 5, 444-452.

Wehner, A. P. (1994). Biological effects of cosmetic talc. Food Chem Toxicol, 32, 1173-1184.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell, 81, 323.

Weiss, N. S.,Lyon, J. L.,Liff, J. M.,Vollmer, W. M., and Daling, J. R. (1981). Incidence of ovarian cancer in relation to the use of oral contraceptives. Int J Cancer, 28, 669-671.

Weissenbach, J.,Gyapay, G.,Dib, C.,Vignal, A.,Morissette, J.,Millasseau, P.,Vaysseix, G., and Lathrop, M. (1992). A second-generation linkage map of the human genome. Nature, 359, 794-801.

Weissman, B. E.,Saxon, P. J.,Pasquale, S. R.,Jones, G. R.,Geiser, A. G., and Stanbridge, E. J. (1987). Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. Science, 236, 175-180.

Welch, D. R.,Chen, P.,Miele, M. E.,McGary, C. T.,Bower, J. M.,Stanbridge, E. J., and Weissman, B. E. (1994). Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. Oncogene, 9, 255-262.

Weston, A.,Willey, J. C.,Modali, R.,Sugimura, H.,McDowell, E. M.,Resau, J.,Light, B.,Haugen, A.,Mann, D. L.,Trump, B. F., and et, a. (1989). Differential DNA sequence deletions from chromosomes 3, 11, 13, and 17 in squamous-cell carcinoma, large-cell carcinoma, and adenocarcinoma of the human lung. Proc Natl Acad Sci U S A, 86, 5099-5103.

Wewer, U. M.,Liotta, L. A.,Jaye, M.,Ricca, G. A.,Drohan, W. N.,Sen, R.,Mudryj, M., and Sobel, M. (1986). Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. Proc Natl Acad Sci, 84, 6725-6729.

Whittemore, A. S.,Wu, M. L.,Paffenbarger, R., Jr.,Sarles, D. L.,Kampert, J. B.,Grosser, S.,Jung, D. L.,Ballon, S., and Hendrickson, M. (1988). Personal and environmental characteristics related to epithelial ovarian cancer. II. Exposures to talcum powder, tobacco, alcohol, and coffee. Am J Epidemiol, 128, 1228-1240.

Whittemore, A. S.,Harris, R., and Itnyre, J. (1992). Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. Am J Epidemiol, 136, 1184-1203.

Whittemore, A. S. (1993). Fertility drugs and risk of ovarian cancer. Hum Reprod, 8, 999-1000.

Wilhelm, S. M.,Collier, I. E.,Kronberger, A.,Eisen, A. Z.,Marmer, B. L.,Grant, G. A.,Bauer, E. A., and Goldberg, G. I. (1987). Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. Proc Natl Acad Sci U S A, 84, 6725-6729.

Williams, M. E.,Swerdlow, S. H., and Meeker, T. C. (1993). Chromosome-t(11-14)(q13-q32) breakpoints in centrocytic lymphoma are highly localized at the bcl-1 major translocation cluster. Leukemia, 7, 1437-1440.

Winqvist, R.,Hampton, G. M.,Mannermaa, G.,Blanco, G.,Alavaikko, M.,Kiviniemi, H.,Taskinen, P. J.,Evans, G. A.,Wright, F. A.,Newsham, I., and Cavenee, W. K. (1995). Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res, 55, 2660-2664.

Wooster, R.,Neuhausen, S. L.,Mangion, J.,Quirk, Y.,Ford, D.,Collins, N.,Nguyen, K.,Seal, S.,Tran, T.,Averill, D.,Fields, P.,Marshall, G.,Narod, S.,Lenoir, G.,Lynch, H.,Feunteun, J.,Devilee, P.,Cornelisse, C.,Menko, F.,Daly, P.,Ormiston, W.,McManus, R.,Pye, C.,Lewis, C.,Cannon-Albright, L.,Peto, J.,Ponder, B.,Skolnick, M.,Easton, D.,Goldgar, D., and Stratton, M. (1994). Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science, 265, 2088-2090.

Wooster, R.,Bignell, G.,Lancaster, J.,Swift, S.,Seal, S.,Mangion, J.,Collins, N.,Gregory, S.,Gumbs, C.,Micklem, G.,Barfoot, R.,Hamoudi, R.,Patel, S.,Rice, C.,Biggs, P.,Hashim, Y.,Smith, A.,Connor, F.,Arason, A.,Gudmundsson, J.,Ficenec, D.,Kelsell, D.,Ford, D.,Tonin, P.,Bishop, D.T.,Spurr, N.K.,Ponder, B.A.J.,Eeles, R.,Peto, J.,Devilee, P.,Cornelisse, C.,Lynch, H.,Narod, S.,Lenoir, G., and Egilsson, V. (1995). Identification of the breast-cancer susceptibility gene BRCA2. Nature, 378, 789-792.

Yamada, H.,Wake, N.,Fujimoto, S.,Barrett, J. C., and Oshimura, M. (1990). Multiple chromosomes carrying tumor suppressor activity for a uterine endometrial carcinoma cell line identified by microcell-mediated chromosome transfer. Oncogene, 5, 1141-1147.

Yang-Feng, T. L.,Li, S.,Han, H., and Schwartz, P. E. (1992). Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. Int J Cancer, 52, 575-580.

Yokota, J.,Tsunetsugu-Yokota, Y.,Battifora, H.,Lefevre, C., and Cline, M. J. (1986). Alterations of myc, myb and ras-Ha proto-oncogenes in cancers are frequent and show clinical
correlation. Science, 231, 261.

Yoshida, A.,Asaga, T.,Masuzawa, C., and Kawahara, S. (1994a). Alteration of tumorigenicity in undifferentiated thyroid carcinoma cells by introduction of normal chromosome 11. J Surg Oncol, 55, 170-174.

Yoshida, M. A.,Shimizu, M.,Ikeuchi, T.,Tonomura, A.,Yokota, J., and Oshimura, M. (1994b). In-vitro growth suppression and morphological change in a human renal-cell carcinoma cell-line by the introduction of normal chromosome-3 via microcell fusion. Mol Carcinog, 9, 114-121.

Zheng, J. P.,Robinson, W. R.,Ehlen, T.,Yu, M. C., and Dubeau, L. (1991). Distinction of low-grade from high-grade human ovarian carcinomas on the basis of losses of heterozygosity on chromosome-3, chromosome-6, and chromosome-11 and her-2/neu gene amplification. Cancer Research, 51, 4045-4051.

Zheng, J. P.,Benedict, W. F.,Xu, H. J.,Hu, S. X.,Kim, T. M.,Velicescu, M.,Wan, M. H.,Cofer, K. F., and Dubeau, L. (1995). Genetic disparity between morphologically benign cysts contiguous to ovarian carcinomas acid solitary cystadenomas. J Natl Cancer Inst, 87, 1146-1153.

Zieman-van der Poel, S.,McCabe, N. R.,Gill, H. J.,Espinosa, R.,Patel, Y.,Harden, A.,Rubinelli, P.,Smith, S. D.,Lebeau, M. M.,Rowley, J. D., and Diaz, M. O. (1991). Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukaemias. Proc. Natl. Acad. Sci. USA, 88, 10735-10739.

Zuckerberg, L. R.,Yang, W. I., and Gadd, M. (1995). Cyclin D1 (prad1) protein expression in breast cancer - approximately 1/3 of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. Mol Pathol, 8, 560.

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# Chromosome 11 allele imbalance and clinicopathological correlates in ovarian tumours

H Gabra[1], L Taylor[2], BB Cohen[2], A Lessels[3], DM Eccles[4], RCF Leonard[1], JF Smyth[1] and CM Steel[2,5]

[1]*ICRF Medical Oncology Unit,* [2]*MRC Human Genetics Unit and* [3]*Department of Pathology, Western General Hospital, Edinburgh;* [4]*CRC Genetic Epidemiology Unit, Southampton University;* [5]*School of Biological and Medical Sciences, University of St. Andrews, UK.*

**Summary** Allele imbalance on chromosome 11 loci in ovarian cancer is a frequent event, suggesting the presence of tumour-suppressor genes for ovarian carcinogenesis on this chromosome. Ten highly polymorphic (CA) repeat microsatellites were used to determine allele imbalance in 60 primary ovarian tumours, including 47 epithelial ovarian cancers (EOCs). Forty EOCs (85%) showed allele imbalance at one or more loci, and in 39 of these (83%) the data suggested subchromosomal deletions: eight of 11p only; six of 11q only; and 25 of both 11p and 11q. Three consensus regions of deletion were indicated at 11p15.5-p15.3, 11q12-q22 and 11q23.3-q24.1. Allele imbalance at the 11q subtelomeric region (D11S912) correlated significantly with adverse survival, while imbalance at 11q14.3 and retention of heterozygosity at 11q22 (close to the site of the progesterone receptor gene) were associated with favourable clinicopathological features. The findings allow development of a preliminary model for the molecular evolution of epithelial ovarian cancer.

**Keywords:** chromosome 11; tumour-suppressor genes; ovarian cancer; loss of heterozygosity

Epithelial ovarian cancer is the main cause of death from gynaecological cancer in British women, primarily because of its late presentation, which carries a poor prognosis despite available treatment modalities. Interest is focusing on the molecular basis of ovarian cancer in order to uncover new and hopefully effective management strategies.

Strong circumstantial evidence for the location of tumour-suppressor genes (TSGs) can be obtained by observed allele imbalance (loss of heterozygosity, LOH) in tumour DNA compared with a matched constitutional DNA specimen at defined chromosomal loci (Ponder, 1988). Minimum consensus regions of allele imbalance may lead to isolation and cloning of these 'deleted' genes, and LOH studies in many tumour types have suggested putative TSGs located on chromosome 11.

In ovarian cancer, cytogenetic analysis has demonstrated partial deletions of chromosome 11 affecting both the long and short arms (Bello and Rey 1990; Pejovic *et al.*, 1992; Jenkins *et al.*, 1993). Frequent LOH has been demonstrated at 11p15 (Lee *et al.*, 1989; Ehlen and Dubeau, 1990; Eccles *et al.*, 1992*a*; Gallion *et al.*, 1992; Vandamme *et al.*, 1992; Viel *et al.*, 1992; Kiechleschwarz *et al.*, 1993), although not all studies have confirmed this high level of loss (Sato *et al.*, 1991; Zheng *et al.*, 1991; Yangfeng *et al.*, 1992). A proximal focus at 11p13 (site of *WT1*) exhibits lower rates of LOH in ovarian cancer (Call *et al.*, 1990; Vandamme *et al.*, 1992; Viel *et al.*, 1992; Bruening *et al.*, 1993). A minority of these studies have proposed a correlation of 11p LOH with poorly differentiated (Zheng *et al.*, 1991; Kiechleschwarz *et al.*, 1993) and more advanced (Viel *et al.*, 1992) tumours. Molecular studies of the proximal 11q region have shown low rates of both LOH and amplification of the 11q13 amplicon in ovarian cancer (Lee *et al.*, 1989; Li *et al.*, 1991; Sato *et al.*, 1991; Viel *et al.*, 1992; Foulkes *et al.*, 1993). In contrast, the only study that has looked specifically at the subtelomeric region of 11q (Foulkes *et al.*, 1993) recorded a high rate of allele imbalance at 11q23.3-qter in a small sample of tumours. The advent of highly polymorphic, well-mapped, microsatellites distributed evenly throughout the genome (Weissenbach et al., 1992; Gyapay *et al.*, 1994) and amenable to polymerase chain reaction (PCR) amplification has allowed rapid LOH analysis using small amounts of DNA (Futreal *et al.*, 1992), which can be derived, if necessary, from archival material.

We have used 10 $(CA)_n$ polymorphic microsatellites to determine allele imbalance on chromosome 11 in ovarian tumours removed from 60 women [47 epithelial ovarian cancers (EOCs), five borderline malignancies, three adenofibromas, two mixed mesodermal tumours, two granulosa cell tumours and one teratoma]. The data have been analysed in relation to clinicopathological findings.

## Materials and methods

### *Clinical specimens*

Fresh primary ovarian tumour tissue from 60 patients was transferred directly to dry ice or liquid nitrogen and stored at −70°C. The normal tissue for comparison was blood in 39 patients and normal regions from formalin-fixed blocks in 21 patients. FIGO staging, histopathology and differentiation state were determined and reviewed in a standardised fashion at a multidisciplinary combined gynaecological oncology clinic. Treatment was in accordance with standard protocols, which consisted of the best possible surgical debulking followed by adjuvant/palliative chemotherapy. Minimum follow-up from diagnosis is 24 months, with median follow-up (by reverse Kaplan-Meier method) of 47 months. All deaths that have occurred have been due to ovarian cancer. Patient characteristics are outlined in Table I.

### *DNA extraction*

DNA from fresh-frozen tissue and blood was extracted by a standard technique as previously described (Eccles *et al.*, 1990). DNA extraction from fixed specimens was performed by cutting 3 × 10 μm sections, dewaxing in xylene, washing in 100% ethanol and desiccating the specimen. Proteinase K (200 μg $ml^{-1}$) digestion was performed overnight at 37°C followed by heat inactivation. Debris was removed by centrifugation, providing a preparation containing adequate DNA template for PCR.

Correspondence: H Gabra, ICRF Medical Oncology Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK
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**Table I** Clinicopathological characteristics of the 60 patients with ovarian tumours

| | |
|---|---|
| Number of patients | 60 |
| *Ovarian adenocarcinoma* | 47 |
| Histology | |
| Serous | 25 |
| Endometrioid | 14 |
| Mucinous | 5 |
| Clear cell | 3 |
| Differentiation | |
| Well | 3 |
| Moderate | 14 |
| Poor | 25 |
| Not known | 5 |
| Stage | |
| I/II | 16 |
| III/IV | 29 |
| Not known | 2 |
| Surgical treatment | |
| Completely debulked | 32 |
| Incompletely debulked | 15 |
| Not known | 2 |
| Chemotherapy | |
| Chlorambucil | |
| Adjuvant | 3 |
| Palliative | 6 |
| *Cis*-platinum | |
| Adjuvant | 11 |
| Palliative | 8 |
| Carboplatinum | |
| Palliative | 2 |
| None | 13 |
| Borderline malignant potential | 5 |
| Mixed mesodermal tumour | 2 |
| Granulosa tumour | 2 |
| Teratoma | 1 |
| Benign adenofibroma | 3 |

*Oligonucleotide primers*

Primers were selected on the basis of recently generated microsatellite index maps for locus, informativeness and spacing. Table II shows these primers and associated information. A high-resolution radiation hybrid map allowed reasonable estimates of physical distance separating these markers (Figure 1) (James *et al.*, 1994).

*Polymerase chain reaction and polymorphic microsatellite detection*

PCR was performed under conditions specified in the original papers. A 10 μl volume of the PCR reaction product was loaded onto 8% denaturing polyacrylamide gel, separated by electrophoresis, passively transferred to Hybond nylon and probed with a $^{32}$P end-labelled poly(CA) probe as previously described (Cohen *et al.*, 1992). Two observers visually analysed the autoradiographs and recorded allele imbalance when there was a clear reduction in the intensity of one allele in tumour DNA.

*Statistical analysis*

The two-tailed Fisher exact test was used. Since numerous analyses were performed, significance was set at $P = 0.01$, but we have included trends towards significance in the region of $.07 > P > 0.01$ where they have supported or suggested biological hypotheses. Kaplan–Meier curves and log-rank analysis were performed (ICRF ICNET PDPLOT actuarial survival program, W Gregory) to determine LOH–survival relationships. Multivariate analysis was not performed because of the small sample number.

**Table II** Polymorphic chromosome 11 microsatellite markers used in this study: identity and location

| *Locus* | *Location* | *Name* | *References*[a] |
|---|---|---|---|
| D11S922 | 11p15.5 | AFM217yb10 | 1,2 |
| D11S569 | 11p15.3 | cCIII-434 | 2,3 |
| D11S929 | 11p14.1 | AFM234xc3 | 1,2 |
| D11S935 | 11p13 | AFM254zb9 | 1,2 |
| D11S905 | 11p13–12 | AFM105xb10 | 1,2 |
| D11S873 | 11q14.3 | Mfd127 | GDB ID no. 32638 |
| D11S35 | 11q22 | Phage2-22 | 2,4 |
| D11S897 | 11q23.1 | Mfd231 | GDB ID no. 34742 |
| D11S925 | 11q23.3 | AFM220yb6 | 1,2 |
| D11S912 | 11q24.1 | AFM157xh6 | 1,2 |

[a]1, Weissenbach *et al.* (1992), Gyapay *et al.* (1994), Couillin *et al.* (1994). 2, Litt *et al.* (1993). 3, Phromchotikul *et al.* (1992). 4, Litt *et al.* (1990). GDB, genome database.

## Results

*Molecular analysis*

Clinicopathological characteristics of the patient cohort are outlined in Table I. Table III shows the allele imbalance results for all markers and subgroups in this study.

Eighty-seven per cent of all ovarian tumours (52/60) and 85% of EOCs (adenocarcinomas excluding borderline malignancies) (40/47) had evidence of LOH involving at least one locus on chromosome 11. Only one EOC had LOH at all informative loci, and seven EOCs (15%) had no detectable LOH. Examples of allele loss for each of the markers are shown in Figure 1.

*Analysis of consensus regions of allele imbalance in ovararian EOCs* Figure 2 is a graphic representation of the data from Table III showing that serous, poorly differentiated and advanced stage EOCs have particularly high levels of LOH at both the 11p and 11q subtelomeric regions. Conversely, EOCs which are early stage or moderately/well differentiated appear to have high levels of LOH at the 11q14.3–q22 region.

Figure 3 shows those tumours that have partial losses on chromosome 11. Deletions are shown in shaded bars and are limited by the next heterozygous locus. In cases where a locus with allele loss is separated by an uninformative locus from a locus that remains heterozygous, that uninformative locus is included within the shaded bars as part of the deletion (since this region could be deleted). Eight tumours had only 11p loss, six tumours had only 11q loss and 25 tumours had partial loss of both arms. This type of analysis suggests three shortest regions of overlap (SROs) corresponding to three consensus regions of deletion/allele imbalance at 11p15.5–15.3, 11q23.3–qter and 11p12–q22.

*11p loss of heterozygosity* LOH was observed for at least one short arm locus in 77% (46/60) of all informative tumours, including 72% of EOCs (34/47).

For all ovarian tumours, high levels of LOH (>40%) were found for three loci (see Table III): D11S922 at 11p15.5 in 24/47 informative tumours (51%) and 16/36 EOCs (44%); D11S569 at 11p15.3 in 23/43 informative tumours (54%) and 14/30 EOCs (47%); and at D11S905 at 11p13–12 in 21/45 informative tumours (47%) and 15/33 EOCs (45%). When considering only those tumours that were informative at both loci telomeric to 11p15.3 (D11S569 and D11S922), the rate of 11p subtelomeric LOH was 16/24 (67%).

The lowest frequencies of allele loss on 11p were detected at D11S929 (11p14.1), with only 28% LOH in ovarian tumours and 24% LOH in EOCs.

*11q loss of heterozygosity* 11q LOH was observed for at least one locus in 65% (39/60) of all informative tumours, including 66% of EOCs (31/47).

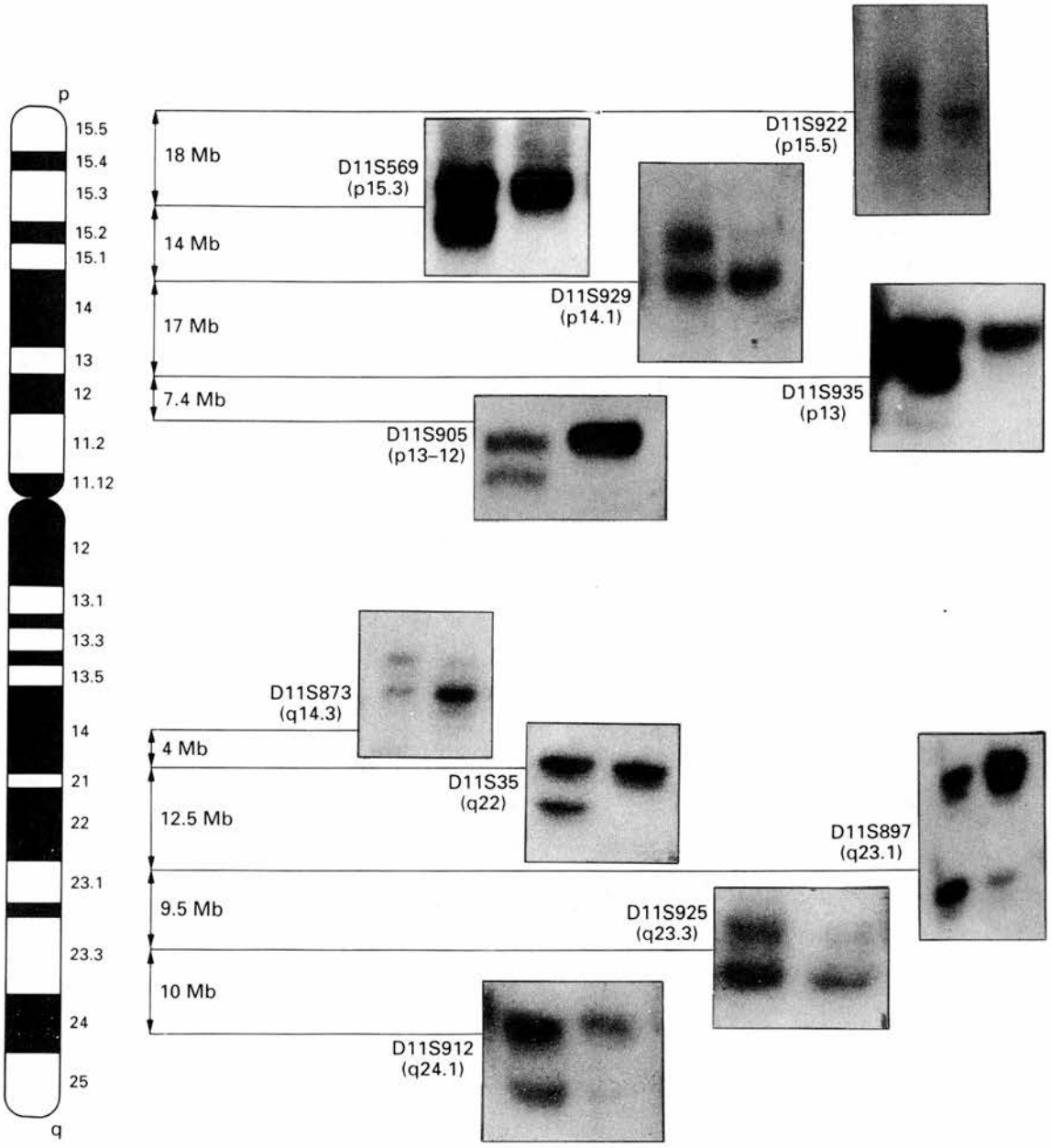


**Figure 1** Idiogram of chromosome 11 with examples of allele imbalance. Constitutive DNA (left lane) and tumour DNA (right lane) for each microsatellite locus are shown with approximate distances between loci.

For all ovarian tumours, high levels of LOH were seen in ree loci (see Table III): D11S873 at 11q14.3 in 11/25 infor- ative tumours (44%) and 9/22 EOCs (41%); D11S925 at q23.3 in 24/45 informative tumours (53%) and 18/33 EOCs 4%); and D11S912 at 11q24.1 in 23/49 informative mours (47%) and 18/37 EOCs (49%).

When considering only those EOCs that were informative both loci telomeric to 11q23.3 (D11S925 and D11S912), e LOH rate was 18/27 (67%).

The lowest frequencies of allele loss were detected at 11S897 (11q23.1), with only 32% LOH in ovarian tumours ıd 28% LOH in EOCs.

*llele imbalance in other ovarian tumour types* The non-OC tumour numbers were too small for statistically valid onclusions. We considered benign and borderline (low aligant potential, LMP) tumours together. Only 1/8 benign borderline tumours had LOH at D11S569 (11p15.3) and 7 had loss at D11S912 (11q24.1). However, 3/5 benign or orderline tumours had LOH at D11S922 (11p15.5). Both e mixed mesodermal tumours had LOH at both 11p15.5–.5.3 and 11q23–qter. One granulosa cell tumour had LOH at all loci on 11p, suggesting whole arm loss. The ovarian teratoma in our series was hemizygous at all nine informative loci, and this is compatible with the usual description of these tumours as being parthenogenetic.

*Microsatellite instability* Microsatellite instability (MSI) (Aaltonen *et al*., 1993; Thibodeau *et al*., 1993) was noted in only 6.4% of EOCs (3/47). Both granulosa cell tumours had evidence of MSI, and one of these tumours had evidence of instability at three loci. There were no cases of MSI in five borderline, three benign and two mixed mesodermal tumours.

### *Statistical analysis*

Fisher's exact test was used to analyse the relationship for allele imbalance between specific loci and also the relationship between imbalance for specific loci and clinicopathological parameters for EOCs.

*Relationship of allele imbalance between different loci* The three regions of deletion determined from Figure 3 were

**Table III** Allele imbalance rates for all subgroups in this study

| *Locus* | *All tumours* | *Adenocarcinoma of ovary* | *Serous* | *Endometrioid* | *Mucinous* | *Early figo* | *Advanced figo* | *Moderately/ well differentiated* | *Poorly differentiated* | *Debulk* | *No debulk* | *Alive* | *Dead* | *Benign/LMP* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D11S922 | 24/47 (51%) | 16/36 (44%) | 11/20 (55%) | 4/11 (36%) | 0/2 | 3/12 (25%) | 11/22 (50%) | 5/13 (38%) | 8/20 (40%) | 10/26 (38%) | 4/8 (50%) | 5/17 (29%) | 10/18 (56%) | 3/5 (60%) |
| D11S569 | 23/43 (54%) | 14/30 (47%) | 9/19 (47%) | 3/5 (60%) | 1/4 (25%) | 4/9 (44%) | 10/20 (50%) | 4/12 (33%) | 10/16 (62%) | 9/20 (45%) | 5/9 (56%) | 5/13 (38%) | 9/16 (56%) | 1/8 (12.5%) |
| D11S929 | 13/47 (28%) | 9/38 (24%) | 3/20 (15%) | 3/11 (27%) | 1/4 (25%) | 3/13 (23%) | 5/23 (21%) | 5/15 (33%) | 3/18 (17%) | 6/26 (23%) | 3/11 (27%) | 4/16 (25%) | 5/21 (24%) | 2/5 (40%) |
| D11S935 | 18/50 (36%) | 13/39 (33%) | 4/18 (22%) | 6/13 (46%) | 2/5 (40%) | 4/15 (27%) | 8/23 (34%) | 6/15 (40%) | 5/20 (25%) | 11/28 (39%) | 2/10 (20%) | 5/19 (26%) | 8/20 (40%) | 1/6 (17%) |
| D11S905 | 21/45 (47%) | 15/33 (45%) | 7/16 (44%) | 5/10 (50%) | 1/5 (20%) | 6/14 (43%) | 9/18 (50%) | 7/12 (58%) | 8/18 (44%) | 13/24 (54%) | 2/8 (25%) | 9/16 (56%) | 6/16 (38%) | 3/7 (43%) |
| D11S873 | 11/25 (44%) | 9/22 (41%) | 5/12 (42%) | 0/5 | 2/3 (66%) | 4/7 (57%) | 5/14 (36%) | 5/7 (71%) | 3/12 (25%) | 6/13 (46%) | 3/8 (37.5%) | 4/7 (57%) | 5/14 (36%) | 1/2 (50%) |
| D11S35 | 16/50 (32%) | 14/39 (36%) | 9/23 (39%) | 0/9 | 2/4 (50%) | 4/11 (36%) | 9/26 (35%) | 4/13 (31%) | 7/21 (33%) | 11/25 (44%) | 4/9 (44%) | 6/17 (35%) | 7/21 (33%) | 1/4 (25%) |
| D11S897 | 13/40 (32%) | 9/32 (28%) | 6/18 (33%) | 1/6 (17%) | 1/4 (25%) | 3/12 (25%) | 6/19 (32%) | 4/13 (31%) | 4/16 (25%) | 5/21 (24%) | 4/10 (44%) | 2/15 (13%) | 7/16 (43%) | 2/5 (40%) |
| D11S925 | 24/45 (53%) | 18/33 (54%) | 11/19 (58%) | 3/8 (38%) | 2/3 (66%) | 2/10 (20%) | 13/21 (62%) | 5/11 (45%) | 10/19 (52%) | 14/22 (64%) | 4/9 (44%) | 6/15 (40%) | 9/17 (53%) | 2/9 (33%) |
| D11S912 | 23/49 (47%) | 18/37 (49%) | 12/21 (57%) | 3/7 (43%) | 2/4 (50%) | 3/13 (23%) | 14/22 (64%) | 5/15 (33%) | 10/18 (56%) | 9/24 (38%) | 8/11 (73%) | 4/17 (27%) | 14/19 (74%) | 1/7 (14%) |

Values given are number of cases with allele imbalance in that subgroup/total number of informative cases in that subgroup (percentage allele imbalance in brackets). Early FIGO = FIGO stage I and II. Advanced FIGO = FIGO stage III and IV. Alive/dead refers to this status at 2 years' minimum follow-up. Debulk = complete debulking at primary operation. No debulk = incomplete debulking at operation. LMP = tumours of low malignant potential (borderline tumours).

analysed for significant relationships. No relationship was noted between LOH at the 11p subtelomeric region (D11S922 and D11S569 at 11p15.5–15.3) and at the 11q subtelomeric region (D11S925 and D11S912 at 11q23.3–24.1) ($P = 0.5$). Similarly, no relationship was seen between LOH at 11p12–q14.3 (D11S905 and D11S873) and the 11p subtelomere ($P = 1.0$). However, a significant relationship exists for LOH at the 11p12–q14.3 region and the 11q subtelomeric region ($P = 0.0025$), suggesting that losses at these two regions co-segregrate. Alternatively, they could be part of a single deletion group, although Figure 3 suggests that this is not the case.

Fisher's exact test was used to test allele imbalance between all possible combinations of loci (Figure 4). For loci adjacent or close to each other, significant and borderline

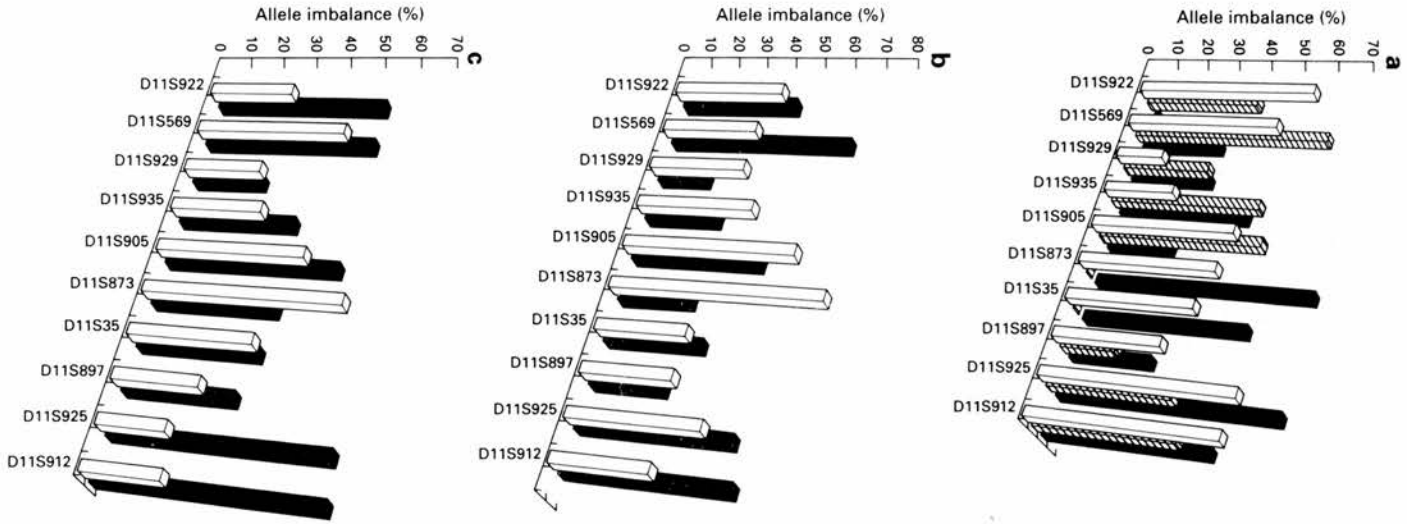


**Figure 2** Graphic representation of allele imbalance rates from Table III for various clinicopathological parameters. **(a)** □, Serous; ▨, endometrioid; ■, mucinous. **(b)** □, Moderately/well differentiated; ■, poorly differentiated. **(c)** □, Early FIGO; ■, advanced FIGO.

findings are likely to reflect association simply as part of substantial subchromosome deletions which may include a tumour-suppressor gene. For loci distant from each other, D11S912/D11S935 LOH showed a significant statistical relationship ($P = 0.0073$) and the relationship for D11S935/D11S922 was of borderline signifiance ($P = 0.046$), suggesting the possibility that these loci harbour genes which may be inactivated cooperatively.

*Relationship between allele imbalance and clinicopathological parameters* Table IV shows Fisher's test *P*-values with significance trends for clinicopathological parameters at the loci tested.

*Allele imbalance and histology* No significant difference was seen at any locus, comparing serous EOCs with other histologies. However, of nine informative endometrioid tumours,

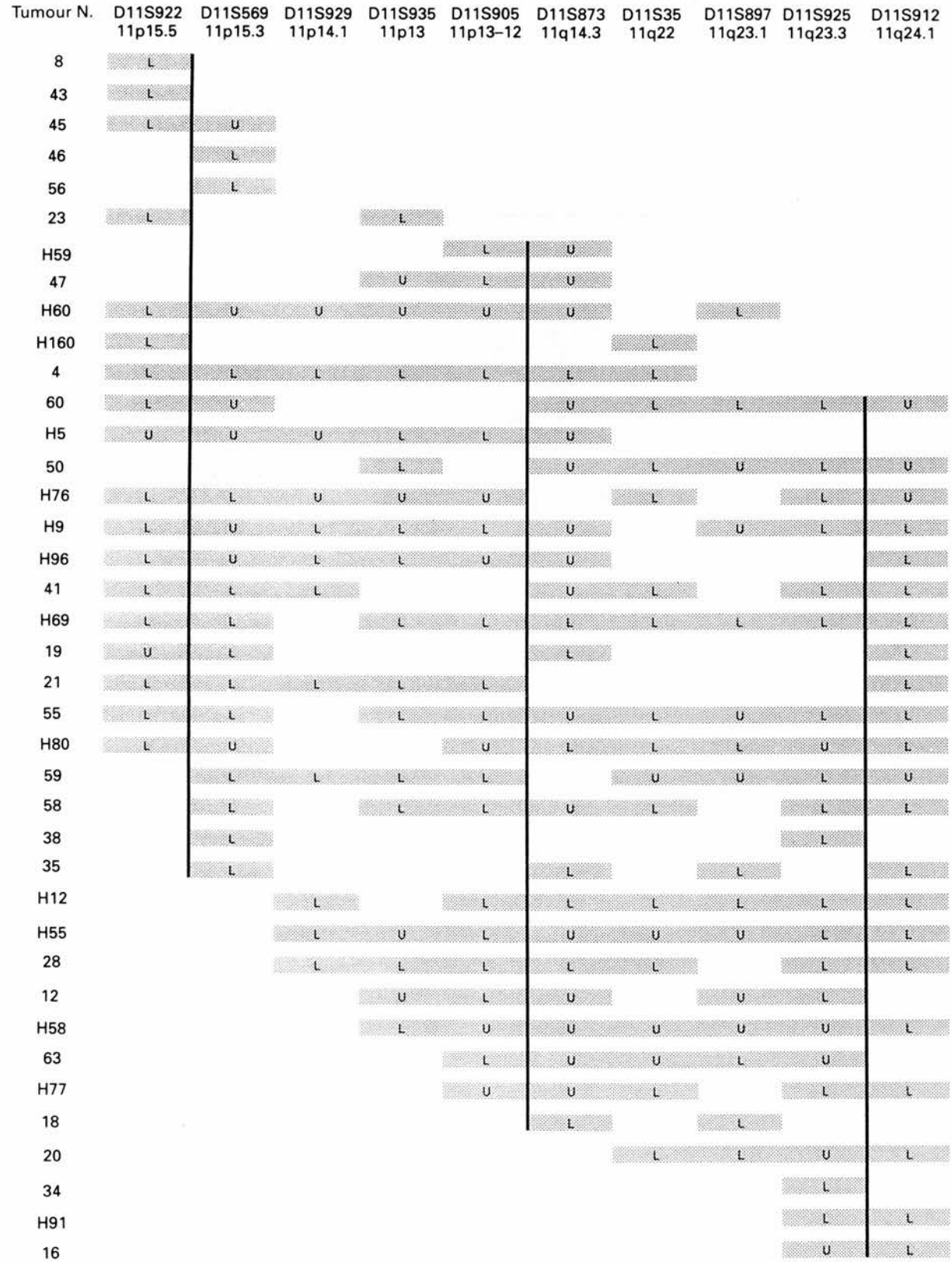


**Figure 3** Grey horizontal bars represent the extent of subchromosomal deletions in EOCs. Black vertical lines represent approximate positions of the shortest regions of overlap (SROs); three such regions are apparent. **L** = constitutively heterozygous with allele loss/imbalance in tumour DNA. **U** = constitutively homozygous and therefore uninformative at that locus.

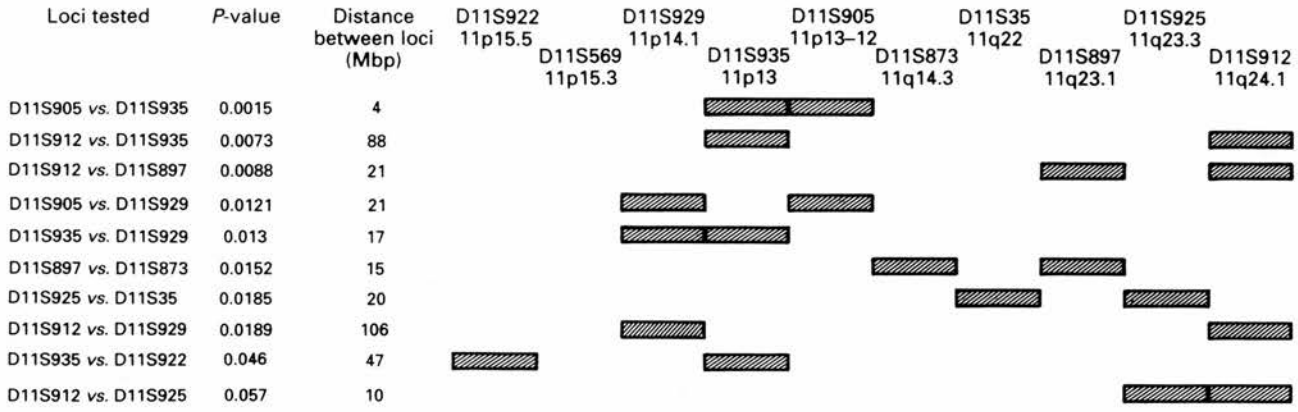


**Figure 4** Fisher's exact test analysis of co-loss between markers on chromosome 11.

**Table IV** Fisher's exact test comparing ovarian adenocarcinoma clinicopathological groups at chromosome 11 loci

| *Marker LOH* | *Segregation parameter* | P-*value* |
|---|---|---|
| D11S912 | Dead (*vs* alive) patients with 24 months minimum follow-up | 0.0067 |
| D11S912 | Dead (*vs* alive) patients at 24 months | 0.067 |
| D11S912 | Late (*vs* early) FIGO stage tumours | 0.035 |
| D11S35 | Non-endometrioid (*vs* endometrioid) histology | 0.04 |
| D11S873 | Well (*vs* poorly) differentiated tumours | 0.07 |
| D11S912 | Non-debulked (*vs* debulked) tumours post surgery | 0.075 |

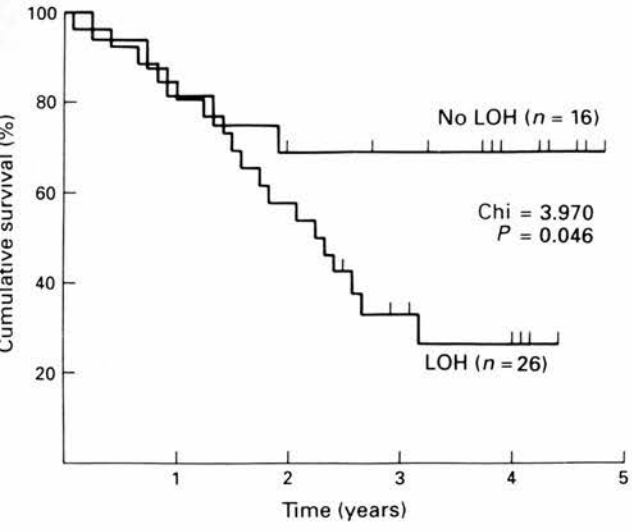


**Figure 5** Kaplan–Meier survival curve with log-rank analysis for subtelomeric 11q allele imbalance status at presentation.

none had LOH at D11S35, and comparing this group with other histologies a trend towards significance was observed for LOH of this marker with non-endometrioid histology ($P = 0.04$).

*Allele imbalance and FIGO stage* The only observed trend towards significance was for the association of LOH at D11S912 with FIGO stage III/IV EOCs ($P = 0.035$).

*Allele imbalance and differentiation grade* The only apparent trend towards significance was between D11S873 LOH and well/moderately differentiated tumours ($P = 0.07$).

*Survival* D11S912 loss of heterozygosity at 11q23.3–24.1 in primary tumours at diagnosis was associated with adverse

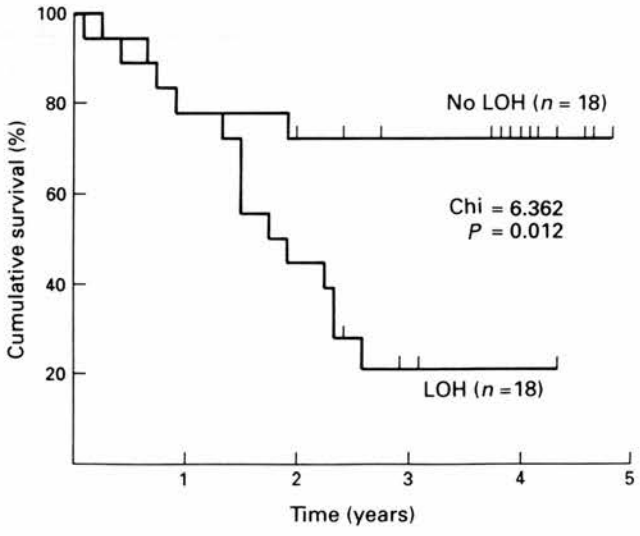


**Figure 6** Kaplan–Meier survival curve with log-rank analysis for D11S912 (11q24.1) allele imbalance status at presentation.

survival of patients with adenocarcinoma ($P = 0.0067$) with minimum follow-up of 24 months.

*Kaplan–Meier survival analysis* Telomeric 11q LOH (D11S925 and D11S912) was significantly associated with adverse survival for patients with ovarian adenocarcinoma (Figure 5), and significance was increased when D11S912 LOH was considered alone (Figure 6). Actuarial survival of those without LOH shows 70% survival at 4 years *vs.* 20% for patients who had lost a D11S912 allele in their primary tumour at diagnosis.

## Discussion

With a panel of ten highly informative, well-distributed, accurately mapped microsatellite polymorphisms (MSPs), significant levels of chromosome 11 allele imbalance were detected in our population of 60 ovarian tumours. Although the term allele imbalance is used interchangeably with LOH in this paper, we have opted to use this term rather than LOH since imbalance can also be a consequence of allele-specific amplification and need not necessarily imply deletion of a region of DNA. Furthermore, amplification of a region of DNA is not mutually exclusive with loss of function at a tumour-suppressor locus; loss of a chromosome or sub-chromosomal region may occur with reduplication of the other allele/chromosome, and amplification of a region of DNA is not necessarily associated with gain of function if accompanying inactivating mutations are involved.

In contrast to findings with chromosome 17 (Steel *et al.*, 1994), in which whole homologue or whole arm loss is common, interstitial and small terminal deletions are in fact more common in chromosome 11 in this same group of ovarian tumours. A highly significant association between allele imbalance on 17p and 17q in this material has been observed previously (Fisher's exact test, $P = 0.0007$; data not presented). No such association is observed for imbalance on 11p and 11q ($P = 0.65$) as a whole, and this argues for caution in the interpretation of allelotyping data that utilise only one or two loci per chromosome arm where no previous biological hypothesis associates that chromosome arm with involvement in neoplasia.

However, relationships for allele imbalance between distant chromosome 11 loci do occur; not only between adjacent sites (which are likely to reflect larger deletions). Significant associations do occur between two distant loci while intervening loci are excluded from the relationship, as shown for example by D11S912/D11S935 and D11S935/D11S922 (at borderline significance). These pairs of loci may harbour genes which are cooperatively inactivated as part of a multistep process. Consensus analysis of those EOCs with partial deletion suggests at least three distinct regions of allele imbalance, at 11p15.5–p15.3, 11q23.3–24.1 and 11p12–q14.3.

In contrast to previous reports (Zheng *et al.*, 1991; Kiechleschwarz *et al.*, 1993), we found no significant association between differentiation grade and allele imbalance at the 11p15 region (despite 67% LOH) in our sample of EOCs, and this may reflect the lack of uniformity in ovarian cancer grading methodology; nor could we confirm an association between 11p15 LOH and advanced stage disease (Viel *et al.*, 1992), although there appeared to be a non-significant trend for both these parameters (Figure 2). D11S922 LOH (11p15.5) did correlate at a borderline level of significance ($P = 0.046$) with LOH at D11S935. (Significant LOH rates have been detected in several studies at 11p13, near the site of *WT*1, although direct analysis of the *WT*1 locus suggests that it is not the gene involved; Bruening *et al.*, 1993; Viel *et al.*, 1994.) There was also evidence of significant LOH at D11S922 in benign and borderline tumours. With this high level of loss in EOCs, and without significant correlations with advanced disease/poor prognosis subgroups, the likelihood is that an 18.6 Mb interval within 11p15 houses a gene involved at an early stage in ovarian carcinogenesis, occurring as part of the development of benign and borderline tumours and also detectable at roughly similar rates in adenocarcinoma. Allele loss at D11S569 (11p15.3) is low (12.5%) in benign and borderline tumours, but is much higher in carcinomas, and there is no difference in LOH rate between early and advanced FIGO stage adenocarcinomas. This raises the possibility of a second locus at 11p15.3 which is inactivated as part of the development of frank adenocarcinoma (albeit at an early stage of adenocarcinoma development).

We have confirmed and extended (in both numbers and chromosomal position) the recent finding of extensive allele loss distal to 11q23.3 (Foulkes *et al.*, 1993), with 67% of EOCs exhibiting LOH at 11q23.3–24.1 in our sample. Our proximal marker (D11S925) in this region maps about 1.2 Mb telomeric to the most distal marker in the study of Foulkes *et al.* Loss of heterozygosity at the distal MSP (D11S912) at 11q24.1 is significantly associated with adverse survival and advanced stage, although the latter *P*-value, at 0.035, is borderline.

No borderline tumours exhibited allele imbalance at D11S912. Allele imbalance at the subtelomeric region at D11S912 showed significant correlation with D11S897 (11q23.1) and D11S935 (11p13 in the region of *WT*1). These findings suggest that a TSG acting primarily as a 'progression suppressor' may be located at 11q23.3–24.1 (or telomeric), and that its inactivation may be a significant late event in the pathogenesis of epithelial ovarian cancer.

The 11p12–q14.3 region (which is a large region containing the centromeric half of 11q), although exhibiting high levels of loss, does not appear to segregate significantly with any particular parameter, although there is a non-significant trend towares LOH in association with better prognosis tumours. Allele imbalance in this region does, however, segregate significantly with imbalance of the 11q subtelomeric region. D11S873 LOH at 11q14–q22 seems to correlate with favourable clinicopathological parameters: higher LOH rates are observed in those with mucinous histology, early FIGO stage and well/moderately differentiated tumours. Higher rates of allele loss at D11S873 are seen in those patients remaining alive (also seen at the neighbouring locus, D11S905 at 11p13–12). These findings suggest the possibility that some well-differentiated, early FIGO stage carcinomas may belong to a genetically distinct subcategory of EOC rather than being simply precursors of aggressive late-stage disease (Figure 7), and that allele loss in the 11p12–q22 region may confer changes incompatible with rapid progression of the disease, e.g. deletion of an oncogene locus essential for tumour progression. It is possible that LOH detected in this region could reflect amplification of the 11q13 region similar to that observed in breast cancer (Karlseder *et al.*, 1994), and we have not ruled out this possibility in the present study, although previous studies of this region in ovarian cancer (Foulkes *et al.*, 1993) suggest that

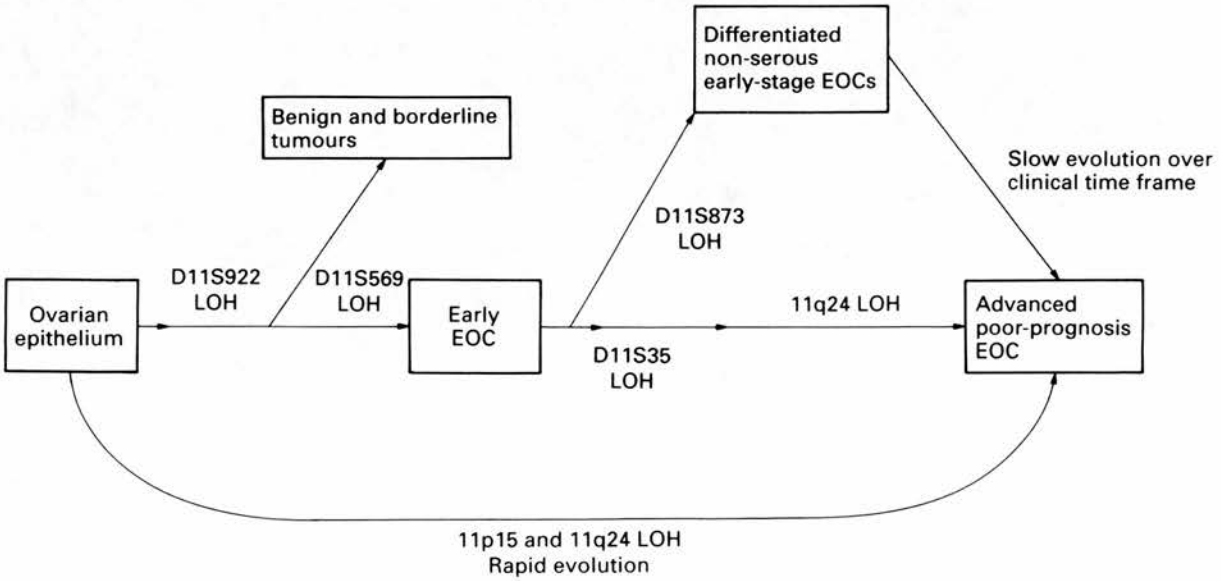


**Figure 7** Hypothesis for chromosome 11 involvement in ovarian carcinogenesis. For abbreviations, see text.

amplification occurs infrequently. However, the explanation for our findings of better prognosis associated with allele loss at this locus remains obscure and requires further work.

The absence of LOH at D11S873 and D11S35 specifically in endometrioid adenocarcinoma is of considerable interest, though the finding approached statistical significance only for the latter MSP ($P = 0.04$). D11S35 lies about 160 kb from the site of the progesterone receptor (PgR) gene. At least six studies have reported that endometrioid tumours contain PgR levels that are elevated relative to other histological types (Slotman and Rao, 1988). Furthermore, there is evidence that, in breast cancer, gene dosage, although secondary to regulatory change, plays a significant role in determining hormone receptor levels: tumours that are cytogenetically 6q−/11q− have half as many oestrogen receptors (ER) and PgRs as tumours without losses on 6q or 11q (Magdelenat *et al.*, 1994). We would therefore speculate that there may be a role for the PgR gene in the regulation of histological subtypes of ovarian cancer, and possibly that its structural disruption contributes to the generation of adverse histological and prognostic subtypes at a relatively early stage in the development of ovarian cancer.

The findings in this study extend the previous observations of distinctive patterns of aneusomy or molecular aberrations in ovarian cancers belonging to different clinicopathological subgroups. They do not imply that LOH at each of the defined regions of chromosome 11 represents independent prognostic factors, although 11q subtelomere imbalance should perhaps be subjected to a large prospective study.

Of more immediate relevance is the application of the observed correlations synthesis of a multistep model of ovarian carcinogenesis (Figure 7). In this model, there is not only multistep, but also multipath, progression of ovarian cancer, postulating, for example, the involvement of q14.3–q22 imbalance in a biologically distinct subpopulation of early EOC.

Extensive chromosome 17 microsatellite and restriction fragment length polymorphism (RFLP) analysis of this same cohort of patients has been performed in our laboratory (Eccles *et al.*, 1992*b*; Steel *et al.*, 1994), and we are currently extending our analysis to correlations between regions of LOH on chromosomes 11 and 17 and their relationship to clinicopathological parameters. In addition, more detailed mapping of LOH within the 11q23.3–qter region to better define the imbalance peak is in progress. This will also determine if there are regions telomeric to 11q24 with low rates of LOH and address the possibility that these observed losses are simply due to non-specific telomeric high-frequency breakages as a consequence of neoplasia rather than putative tumour-suppressor genes at these sites.

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**References**

AALTONEN LA, PELTOMAKI P, LEACH FS, SISTONEN P, PYLKKANEN L, MECKLIN JP, JARVINEN H, POWELL SM, JEN J, HAMILTON SR, PETERSEN GM, KINZLER KW, VOGELSTEIN B AND DE LA CHAPELLE A. (1993). Clues to the pathogenesis of familial colorectal cancer. *Science*, **260,** 812–816.

BELLO MJ AND REY JA. (1990). Chromosome aberrations in metastatic ovarian cancer: relationship with abnormalities in primary tumours. *Int. J. Cancer*, **45,** 50–54.

BRUENING W, GROS P, SATO T, STANIMIR J, NAKAMURA Y, HOUSMAN D AND PELLETIER J. (1993). Analysis of the 11p13 Wilms tumor suppressor gene (wt1) in ovarian tumors. *Cancer Invest.*, **11,** 393–399.

CALL KM, GLASER T, ITO CY, BUCKLER AJ, PELLETRIER J, HABER DA, ROSE EA, KRAL A, YEGER H, LEWIS WH, JONES C AND HOUSEMAN DE. (1990). Isolation and characterisation of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell*, **60,** 509–520.

COHEN BB, WALLACE MR AND CRICHTON DN. (1992). A comparison of procedures for analysing microsatellite repeat polymorphisms. *Mol. Cell. Probes*, **6,** 439–442.

COUILLIN P, LEGUERN E, VIGNAL A, FIZAMES C, RAVISE N, DELPORTES D, REGUIGNE I, ROSIER M, JUNIEN C, VANHEYNINGEN V AND WEISSENBACH J. (1994). Assignment of 112 microsatellite markers to 23 chromosome-11 subregions delineated by somatic hybrids – comparison with the genetic map. *Genomics*, **21,** 379–387.

ECCLES D, CRANSTON G, STEEL CM, NAKAMURA Y AND LEONARD RCF (1990). Allele losses on chromosome 17 in human epithelial ovarian cancer. *Oncogene*, **5,** 1599–1601.

ECCLES DM, GRUBER L, STEWART M, STEEL CM AND LEONARD RCF. (1992*a*). Allele loss on chromosome-11p is associated with poor survival in ovarian cancer. *Dis. Markers*, **10,** 95–99.

ECCLES DM, RUSSELL S, HAITES NE, ATKINSON R, BELL DW, GRUBER L, HICKEY I, KELLY K, KITCHENER H AND LEONARD R. (1992*b*). Early loss of heterozygosity on 17q in ovarian cancer. *Oncogene*, **7,** 2069–2072.

EHLEN T AND DUBEAU L. (1990). Loss of heterozygosity on chromosomal segments 3p, 6q and 11p in human ovarian cancer. *Oncogene*, **5,** 219–223.

FOULKES WD, CAMPBELL IG, STAMP G AND TROWSDALE J. (1993). Loss of heterozygosity and amplification on chromosome 11q in human ovarian cancer. *Br. J. Cancer*, **67,** 268–273.

FUTREAL P, SODERKVIST P, MARKS JR, IGLEHART JD, COCHRAN C, BARRETT JC AND WISEMAN RW. (1992). Detection of frequent allelic loss on proximal chromosome 17 in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res.*, **52,** 2624–2627.

GALLION HH, POWELL DE, MORROW JK, PIERETTI M, CASE E, TURKER MS, DEPRIEST PD, HUNTER JE AND VANNAGELL JR. (1992). Molecular genetic changes in human epithelial ovarian malignancies. *Gynecol. Oncol.*, **47,** 137–142.

GYAPAY G, MORISSETTE J, VIGNAL A, DIB C, FIZAMES C, MILLASSEAU P, MARC S, BERNARDI G, LATHROP M AND WEISSENBACH J. (1994). The 1993–94 Genethon human genetic linkage map. *Nature Genet.*, **7,** 246–300.

JAMES MR, RICHARD III CW, SCHOTT J-J, YOUSTRY C, CLARK K, BELL J, TERWILLIGER JD, HAZAN J, DUBAY C, VIGNAL A, AGRAPART M, IMAI T, NAKAMURA Y, POLYMEROPOULOUS M, WEISSENBACH J, COX DR AND LATHROP GM. (1994). A radiation hybrid map of 506 STS markers spanning human chromosome 11. *Nature Genet.*, **8,** 70–76.

JENKINS RB, BARTELT D, STALBOERGER P, PERSONS D, DAHL RJ, PODRATZ K, KEENEY G AND HARTMANN L. (1993). Cytogenetic studies of epithelial ovarian carcinoma. *Cancer Genet. Cytogenet.*, **71,** 76–86.

KARLSEDER J, ZEILLINGER R, SCHNEEBERGER C, CZERWENKA K, SPEISER P, KUBISTA E, BIRNBAUM D, GAUDRAY P AND THEILLET C. (1994). Patterns of DNA amplification at band-q13 of chromosome-11 in human breast-cancer. *Genes Chrom. Cancer*, **9,** 42–48.

KIECHLESCHWARZ M, BAUKNECHT T, WIENKER T, WALZ L AND PFLEIDERER A. (1993). Loss of constitutional heterozygosity on chromosome-11p in human ovarian-cancer – positive correlation with grade of differentiation. *Cancer*, **72,** 2423–2432.

LEE JH, KAVANAGH JJ, WHARTON JT, WILDRICK DM AND BLICK M. (1989). Allele loss at the c-Ha-ras1 locus in human ovarian cancer. *Cancer Res.*, **49,** 1220–1222.

LI SB, SCHWARTZ PE, LEE WH AND YANGFENG TL. (1991). Allele loss at the retinoblastoma locus in human ovarian cancer. *J. Natl Cancer Inst.*, **83,** 637–640.

LITT M, KRAMER P, HAUGE XY, WEBER JL, WANG Z, WILKIE PJ, HOLT MS, MISHRA S, DONISKELLER H AND WARNICH L. (1993). A microsatellite-based index map of human chromosome-11. *Hum. Mol. Genet.*, **2,** 909–913.

MAGDELENAT H, GERBAULT-SEUREAU M AND DUTRILLAUX B. (1994). Relationship between loss of estrogen and progesterone receptor expression and of 6q and 11q chromosome arms in breast cancer. *Int. J. Cancer*, **57,** 63–66.

PEJOVIC T, HEIM S, MANDHAL N, BALDETORP B, ELMFORS B, FLODERUS U-M, FURGYIK S, HELM G, HIMMELMAN A, WILLEN H & MITELMAN F. (1992). Chromosome aberration in 35 primary ovarian carcinomas. *Genes Chrom. Cancer*, **4,** 58–68.

PONDER B. (1988). Gene losses in human tumours. *Nature*, **335,** 400–402.

SATO T, SAITO H, MORITA R, KOI S, LEE JH AND NAKAMURA Y. (1991). Allelotype of human ovarian cancer. *Cancer Res.*, **51,** 5118–5122.

SLOTMAN BJ AND RAO BR. (1988). Ovarian cancer (review). *Anticancer Research*, **8,** 417–434.

STEEL CM, ECCLES DM, GRUBER L, WALLACE M, LESSELS A., MORSMAN JM, GABRA H, LEONARD RCF, AND COHEN BB. (1994). Allele losses on chromosome 17 in ovarian tumours. In *Ovarian Cancer*, Vol. 3, Sharp F, Mason P, Blackett T and Berek J (eds) pp. 45–52. Chapman & Hall Medical: London.

THIBODEAU SN, BREN G AND SCHAID D. (1993). Microsatellite instability in cancer of the proximal colon. *Science*, **260,** 816–819.

VANDAMME B, LISSENS W, AMFO K, DESUTTER P, BOURGAIN C, VAMOS E AND DEGREVE J. (1992). Deletion of chromosome 11p13–11p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. *Cancer Res.*, **52,** 6646–6652.

VIEL A, GIANNINI F, TUMIOTTO L, SOPRACORDEVOLE F, VISENTIN MC AND BOIOCCHI M. (1992). Chromosomal localization of 2 putative 11p oncosuppressor genes involved in human ovarian tumors. *Br. J. Cancer*, **66,** 1030–1036.

VIEL A, GIANNINI F, CAPOZZI E, CANZONIERI V, SCARABELLI C, GLOGHINI A AND BOIOCCHI M. (1994). Molecular mechanisms possibly affecting wt1 function in human ovarian tumors. *Int. J. Cancer*, **57(4),** 515–521.

WEISSENBACH J, GYAPAY G, DIB C, VIGNAL A, MORISSETTE J, MILLASSEAU P, VAYSSEIX G AND LATHROP M. (1992). A second-generation linkage map of the human genome. *Nature*, **359,** 794–801.

YANGFENG TL, LI SB, HAN H AND SCHWARTZ P. (1992). Frequent loss of heterozygosity on chromosome-xp and chromosome-13q in human ovarian cancer. *Int. J. Cancer*, **52,** 575–580.

ZHENG JP, ROBINSON WR, EHLEN T, YU MC AND DUBEAU L. (1991). Distinction of low-grade from high-grade human ovarian carcinomas on the basis of losses of heterozygosity on chromosome-3, chromosome-6, and chromosome-11 and her-2/neu gene amplification. *Cancer Res.*, **51,** 4045–4051.

*Advances in Brief*

# Loss of Heterozygosity at 11q22 Correlates with Low Progesterone Receptor Content in Epithelial Ovarian Cancer

**Hani Gabra,[1] Simon P. Langdon, J. E. Vivienne Watson, R. Anthony Hawkins, Brian B. Cohen, Lorna Taylor,[2] James Mackay, C. Michael Steel, Robert C. F. Leonard, and John F. Smyth**

Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU [H. G., S. P. L., J. E. V. W., L. T., J. M., R. C. F. L., J. F. S.]; Department of Medical Sciences, University of St. Andrews [B. B. C., C. M. S.]; and Department of Surgery, Royal Infirmary of Edinburgh, Edinburgh [R. A. H.], United Kingdom

## Abstract

**Forty-seven epithelial ovarian cancers were analyzed for loss of heterozygosity (LOH) at *D11S35* (11q22), close to the progesterone receptor (*PR*) gene, and for tumoral estrogen receptor (ER) and PR content. Thirty-eight of 47 tumors were informative, and, of these, 14 exhibited LOH. There was a significant association ($P = 0.014$) between *D11S35* LOH and low tumoral PR content. For all informative tumors, there was no correlation between ER and PR; however, exclusion of tumors with LOH from the informative series revealed a linear correlation between tumoral ER and PR ($P = 0.013$), and established ER ($P = 0.025$) and PR ($P = .05$) content as significant factors in relation to patient survival. Patients with ER-rich tumors with *D11S35* LOH had particularly poor survival compared with ER-rich, *D11S35* heterozygous, no loss patients ($P = 0.014$).**

**Analysis of the same tumors using two other microsatellites, *D11S935* (11p13) and NM23 (17q22), showed no statistically significant relationships, although there were nonsignificant trends for the correlation of ER and PR expression in informative tumors without allele loss at these loci.**

**We propose that genomic structural alteration at or close to the *PR* gene locus has biological and clinical sequelae in ovarian cancer.**

## Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy, and its etiology is poorly understood. Epidemiological and animal studies indicate that ovarian cancer may be an endocrine-related tumor. Chronic estrogen and progestin administration in animal studies results in ovarian cancer (1, 2). "Incessant ovulation" appears to be a major premenopausal risk factor (3, 4). Nulliparity and a low mean number of pregnancies have been associated with increased risk (5). Increased total pregnancy and lactation time, and the use of oral contraceptives (6, 7) confer a protective effect. The high gonadotrophic milieu of the postmenopausal state may confer additional risk (8). Ovarian cancer has been reported to respond to antiestrogens in about 10–20% of cases and to progestins with an average of 36%, with a range between 0 and 60% (9, 10).

The ovary is the main site of synthesis of estrogen and progesterone, and is also a target organ for these hormones. The actions of these hormones are mediated by specific intracellular receptors that function as hormone-inducible nuclear transcription factors with context-specific, often conflicting effects on proliferation and differentiation of target tissues (11).

PR[3] is regulated by estrogen via ER (12); estradiol-induced PR expression has been demonstrated in ovarian cancer cell lines that express ER (13).

That histopathological subtypes of epithelial ovarian cancer differ in their PR expression is suggested by at least six reports demonstrating that endometrioid ovarian tumors contain relatively more PR than other histological types (9), and that PR positivity is associated with well-differentiated ovarian tumors in premenopausal women (14). High PR content in breast (15) and ovarian (16, 17) cancer has been shown to correlate with a favorable prognosis, although the biological relevance of steroid hormones and their receptors to ovarian cancer remains controversial.

The *PR* gene has been localized to chromosome 11q22 (18, 19), so that it is now possible to determine whether structural or regulatory disturbances of the chromosomal locus affect the PR content of endocrine-regulated epithelial cancers and hence these tumors' relationships with prognosis. In breast cancer, allele losses on chromosome 11p were found to correlate with PR-negative tumors (20). Despite initial studies suggesting that 11q LOH is an infrequent event (21, 22), several recent studies suggest that it is, in fact, a common event in breast cancer (23–25). Furthermore, a recent breast cancer cytogenetic study (26) demonstrated that 11q- but not 6q- tumors contained a low PR:ER ratio, whereas 6q- but not 11q- tumors had a low ER:PR ratio, suggesting some role for genomic structural alteration in the regulation of tumoral steroid receptor content.

In ovarian cancer, evidence has accrued to suggest that distal chromosome 11q cytogenetic abnormalities (27) and LOH (28, 29) are frequent events of clinicopathological significance.

In this study, we used a polymorphic microsatellite lying close to the *PR* gene to determine allele loss in ovarian cancer. At the time of the study, no intragenic microsatellite with high heterozygosity was available for the *PR* gene, RFLP analysis for the *PR* gene being even less informative. *D11S35* was selected

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[2] To whom requests for reprints should be addressed.
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[3] The abbreviations used are: PR, progesterone receptor; ER, estrogen receptor; LOH, loss of heterozygosity; EIA, enzyme immunoassay.

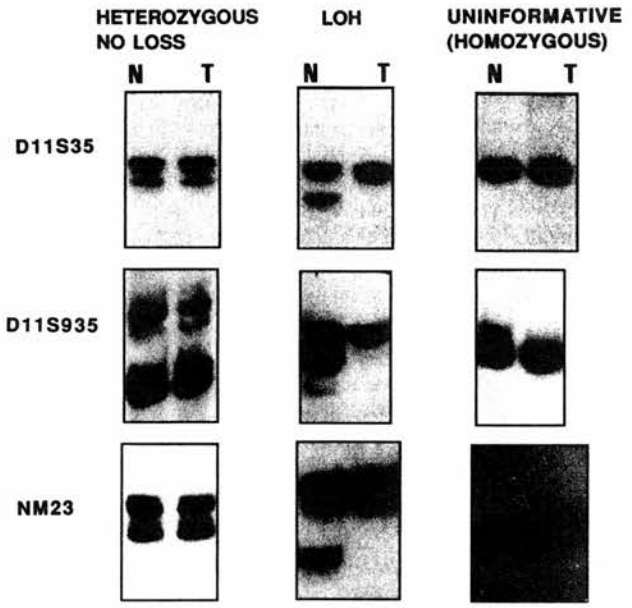


*Fig. 1* Examples of heterozygosity, homozygosity, and allele loss by PCR analysis of the three polymorphic microsatellites used. *N*, PCR from normal constitutive DNA; *T*, tumor DNA. Microsatellite markers map to 11q22 (*D11S35*), 11p13 (*D11S935*), and 17q22 (NM23).

because it was the closest microsatellite to the *PR* gene (with the same Kosambi genetic distance of 0.41) and had an acceptably high heterozygosity rate (30, 31). Subsequent to completing the study, a high resolution physical map of chromosome 11 suggested that it lay slightly (3 centirays, approximately 150 kb) telomeric to the *PR* gene (32). Two other incidental polymorphic microsatellites were randomly selected to demonstrate specificity of the correlation: *D11S935* (11p13) (33) and NM23 (17q22) (34). The PR and ER content of these tumors was then measured, and the relationship between allele loss and tumoral steroid receptor content was assessed.

## Materials and Methods

**Clinical Specimens.** Fresh primary ovarian tumor tissue from 47 patients (mean age, 60 years) with epithelial ovarian cancer was transferred directly to dry ice or liquid nitrogen perioperatively, and stored at −70°C until processing. Heparinized blood was obtained from 34 patients postoperatively. In the remaining 13 patients, normal tissue was obtained from formalin-fixed blocks. The International Federation for Gynecology and Obstetrics staging, histopathology, and differentiation state were determined and reviewed in a standardised fashion at a multidisciplinary combined Gynecological Oncology Clinic. Treatment was planned and delivered in accordance with standard protocols, which consisted of the best possible surgical debulking followed by adjuvant/palliative chemotherapy where appropriate. Minimum follow-up on living patients is 24 months, maximum follow-up is 60 months, and all deaths that have occurred have been due to ovarian cancer.

Numbers of patients with tumors that were ultimately informative for steroid receptor content and constitutive heterozygosity for the microsatellite used. Patient characteristics are outlined in Table 1.

**DNA Extraction.** DNA from fresh frozen tissue was extracted using a standard technique as described previously (35). Extraction of DNA from fixed specimens was performed by cutting 3- × 10-μm sections from the block, dewaxing in xylene for 30 min twice, removing the xylene by washing three times in 100% ethanol, and desiccating the specimen under heat. Proteinase K (200 μg/ml) digestion was performed overnight at 37°C, and then the proteinase K was heat inactivated at 95°C for 5 min and debris was removed by centrifugation. The resultant preparation provided adequate DNA template for PCR.

**Oligonucleotide Primers.** The oligonucleotide primer sequences for *D11S35* (30) were S35-S (5′-ACAATTGGATTACTACTAGCACC-3′) and S35-AS (5′-AACTATGTATTTGTATCGATTAAC C-3′). For *D11S935* (33), the primer sequences were afm 254zb9-CA (5′-TACTAACCAAAAGAGTTGGGG-3′) and afm 254zb9-GT (5′-CTATCATTCAGAAAATGTTGGC-3′). The primer sequences for NM23 (34) were (5′-TTGACCGGGGTAGAGAACTC-3′) and (5′-TCTCAGTACTTCCCGTGACC-3′).

**PCR and Polymorphic Microsatellite Detection.** PCR was performed in a reaction volume of 50 μl under the following reaction conditions: 94°C for 3 min × 1 cycle, 94°C for 1 min/55°C for 1 min/72°C for 1 min × 35 cycles, and 72°C for 5 min × 1 cycle in a Hybaid thermocycler. Reaction mix contained 50 mM KCl, 10 mM Tris-HCl (pH 9), 1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphate, 1.25 units *Taq* polymerase, 10 pmol of each primer, and 100 ng DNA.

Ten μl of the PCR reaction product were loaded onto 8% denaturing polyacrylamide gel, separated by electrophoresis, passively transferred to Hybond nylon, and probed with a $^{32}P$ end-labeled poly(CA) probe as previously described (36). Two observers visually analyzed the autoradiographs and recorded allele imbalance when there was clear reduction in the intensity of one allele in the tumor DNA.

**Determination of ER and PR by EIA.** Tumor specimens snap frozen at operation were homogenized in buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 8.0, plus 1% v/v monothiolglycerol and 10% v/v glycerol) as previously described (37). After centrifugation at 105,000 × *g*, the supernatant cytosol was assayed using EIA kits according to the manufacturer's method (ER-EIA and PR-EIA kits; Abbott Laboratories, Maidenhead, Berkshire, United Kingdom; Ref. 38). The protein content of the cytosol was determined according to the method of Bradford (39), and receptor concentrations were expressed as fmol/mg protein. The PR antibody used was Kd68, a rat mAb which recognizes both the A and B subunits of PR (and therefore recognizes sequences downstream of codon 165, the start site for subunit A) and binds both free and complexed PR, presumably avoiding the hormone-binding site (40).

Recognizing that cutoff values for steroid receptor levels are controversial, ER poor or PR poor was defined as less than 30 fmol/mg protein and ER rich or PR rich was defined as 30 fmol/mg protein or more. These values derive from reports describing clonogenic assays of human ovarian carcinoma cells

*Table 1* Clinicopathological parameters of the patient series

| Tumor | Survival (mo) | Alive ? | Stage (FIGO)[a] | Histology | Grade | *D11S35* 11q22 | NM23 17q22 | *D11S935* 11p13 | PR (fmol/mg protein) | ER (fmol/mg protein) |
|---|---|---|---|---|---|---|---|---|---|---|
| G3 | 9 | | 3 | Serous | P.D. | H | H | H | 159 | 29 |
| G4 | 58 | A | 1 C | Clear cell | M.D. | L | U | L | 2 | 5 |
| G8 | 56 | A | 2 | Serous | P.D. | H | L | U | 100 | 12 |
| G11 | 27 | | 3 | Serous | M.D. | U | U | L | 100 | 12 |
| G12 | 5 | | 4 | Serous | P.D. | H | L | U | 1 | 1 |
| G16 | 21 | | 3 | Mucinous | NK | H | L | H | 1 | 1 |
| G17 | 52 | A | 3 | Serous | M.D. | U | U | H | 2 | 3 |
| G18 | 11 | | 1 A | Mucinous | M.D. | H | L | H | 29 | 8 |
| G20 | 23 | | 3 | Serous | P.D. | L | H | H | 4 | 229 |
| G23 | 10 | | 3 | Endometrioid | M.D. | H | U | L | 21 | 9 |
| G24 | 51 | A | 3 | Serous | P.D. | H | L | H | 2 | 160 |
| G26 | 7 | | 3 | Serous | P.D. | H | U | H | 38 | 39 |
| G28 | 52 | A | 1 A | Mucinous | M.D. | L | H | L | 7 | 6 |
| G30 | 23 | | 1 A | Endometrioid | P.D. | H | U | H | 12 | 12 |
| G34 | 49 | A | 3 B | Serous | P.D. | H | L | H | 187 | 163 |
| G35 | 1 | | 3 | Serous | P.D. | H | U | H | 6 | 12 |
| G38 | 48 | A | 1 A | Endometrioid | M.D. | H | H | H | 139 | 161 |
| G42 | 47 | A | 1 B | Endometrioid | P.D. | H | L | H | 9 | 10 |
| G46 | 46 | A | 1 A | Mucinous | W.D. | U | II | H | 1144 | 315 |
| G50 | 48 | A | 3 | Mucinous | NK | L | H | L | 11 | 10 |
| G55 | 11 | | 4 | Serous | P.D. | L | L | L | 3 | 10 |
| G56 | 43 | A | 1 A | Endometrioid | P.D. | H | L | H | 6 | 11 |
| G58 | 18 | | 3 | Serous | P.D. | L | L | L | 6 | 19 |
| G59 | 38 | | 3 | Endometrioid | P.D. | U | L | L | 74 | 109 |
| G60 | 30 | | 1 C | Serous | P.D. | L | L | H | 11 | 40 |
| H5 | 38 | A | 1 C | Endometrioid | P.D. | U | H | L | 8 | 18 |
| H9 | 37 | A | 3 | Endometrioid | M.D. | H | H | L | 130 | 59 |
| H12 | 35 | A | 1 C | Clear cell | M.D. | L | H | H | 3 | 7 |
| H55 | 8 | | 3 | Serous | P.D. | U | H | U | 10 | 8 |
| H58 | 31 | | 1 | Endometrioid | M.D. | U | L | L | 4 | 116 |
| H59 | 33 | A | 1 | Endometrioid | M.D. | H | U | H | 99 | 30 |
| H60 | 16 | | 3 | Endometrioid | W.D. | H | L | U | 13 | 10 |
| H69 | 25 | | 3 | Serous | P.D. | L | L | L | 5 | 35 |
| H76 | 25 | | 3 | Serous | P.D. | L | L | U | 3 | 88 |
| H77 | 27 | | 3 | Clear cell | M.D. | L | L | H | 3 | 48 |
| H80 | 18 | | 3 | Serous | NK | L | L | H | 4 | 46 |
| H91 | 28 | | 3 | Serous | P.D. | H | H | H | 3 | 21 |
| H95 | 24 | A | 1 C | Endometrioid | P.D. | U | H | H | 42 | 21 |
| H96 | 9 | | ? | Endometrioid | NK | H | L | L | 21 | 8 |

[a] FIGO, International Federation of Gynecology and Obstetrics; A, alive at most recent analysis; U, uninformative/homozygous; L, LOH; H, heterozygous with no loss; NK, not known.

clinical work showing that these approximate cutoff values for ER and PR represent a reasonable index of hormone sensitivity (13, 41, 42).

**Statistical Analysis.** The Mann-Whitney *U* test was used to compare the means and medians of tumor PR content in populations with and without LOH for the three microsatellite markers. Two-tailed *P* values were calculated.

Spearman's rank correlation was used to examine correlations between tumor ER and PR content in those with and without LOH.

Kaplan-Meier/log rank analysis was performed to determine any relationship between the allele loss and survival in *D11S35* informative subgroups.

## Results

**Allele Loss Analysis.** Fig. 1 shows examples of the three microsatellite markers used in this study. Of 47 epithelial ovarian cancer patients, 38 were informative (constitutively heterozygous) at *D11S35* (72%). Of these, 14 (37%) exhibited LOH at this locus and 24 retained heterozygosity. There was inadequate tissue for ER and PR quantification in seven of these informative specimens (two with LOH and five heterozygous, no loss), and therefore the final allele loss rate in this group was 12 (39%) of 31. For *D11S935*, the final allele loss rate was 13 (39%) of 33 and for NM23 (17q22), the final allele loss rate was 19 (61%) of 31 (Table 1). The sample contained nine informative endometrioid ovarian cancers, none of which exhibited LOH at *D11S35*. Fisher's exact test for endometrioid *versus* other histologies revealed a significant correlation between *D11S35* retention of heterozygosity and endometrioid histology ($P = 0.04$) in the 38 informative tumors.

**PR Distribution and Relationships between Allele Loss and PR.** Sufficient material was available for ER and PR measurement in 39 of the 47 tumors (Table 1), and this estimation was performed in a blind fashion at a different institution (by R. A. H.). A summary of PR data distribution by locus and allele loss status is shown in Table 2. The mean tumoral PR concentration for the 12 patients with LOH at *D11S35* was 5.2

*Table 2* Distribution of PR data by allele loss status for three microsatellites[a]

| | *D11S35* 11q22 | | | *D11S935* 11p13 | | | NME-1 17q22 | |
|---|---|---|---|---|---|---|---|---|
| | LOH | HET[b] | LOH | HET | | LOH | HET | |
| No. | 12 | 19 | 13 | 20 | | 19 | 12 | |
| Mean PR (fmol/mg protein) | 5.2 | 51 | 30 | 93.2 | | 25 | 138 | |
| Median PR (fmol/mg protein) | 4 | 21 | 8 | 7.5 | | 6 | 10.5 | |
| SD | 3.1 | 62 | 43 | 254 | | 47 | 322 | |
| Minimum | 2 | 1 | 2 | 1 | | 1 | 3 | |
| Maximum | 11 | 187 | 130 | 1144 | | 187 | 1144 | |
| Mann-Whitney *U* statistic | 53 | | 125 | | | 76.5 | | |
| Two-tailed *P* | 0.014 | | 0.87 | | | 0.133 | | |

[a] Associated nonparametric statistics compare the LOH with heterozygous/no loss groups.
[b] HET, heterozygous.

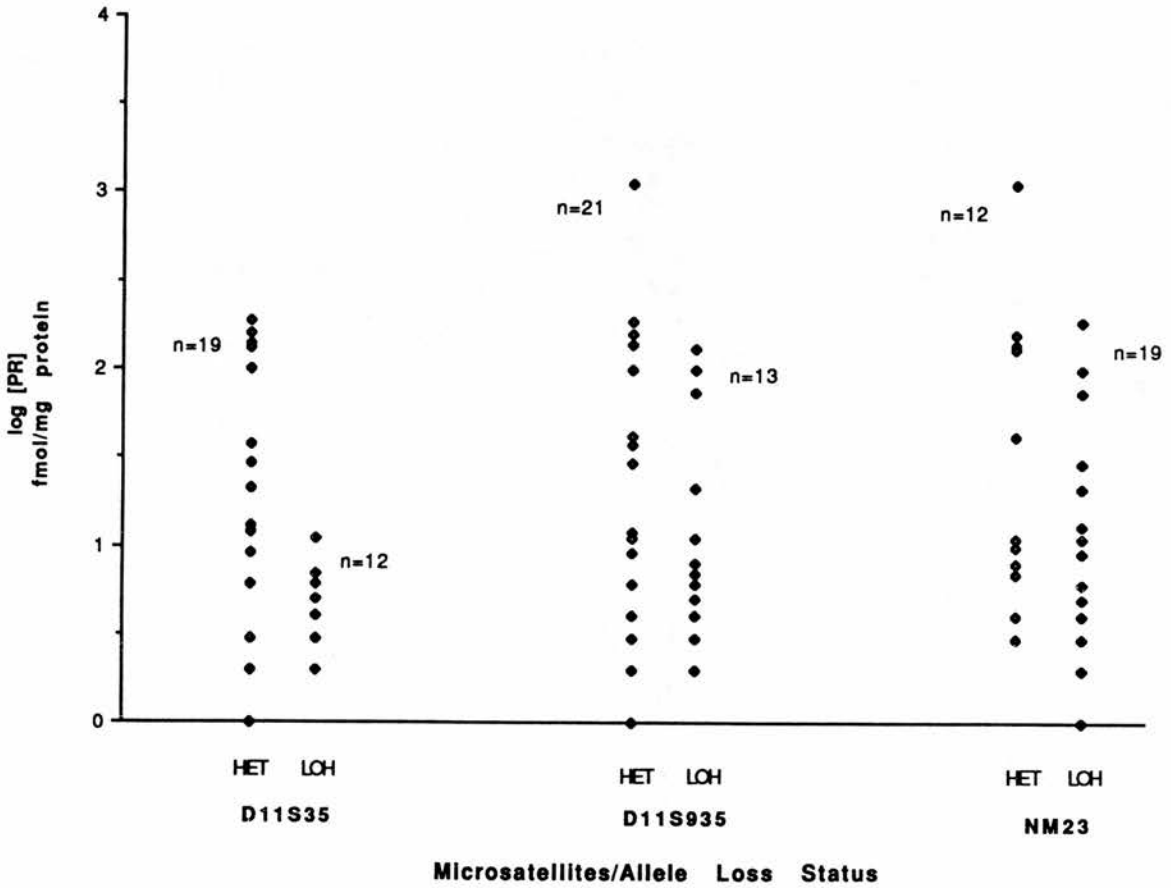


*Fig. 2* Tumoral PR content ($\log_{10}$ transformed values) plotted against tumoral allele loss status for the three microsatellites used. *HET*, heterozygous at the locus without LOH.

fmol/mg, with a median of 4 fmol/mg (range, 2–11 fmol/mg). The mean tumoral PR concentration for the 19 heterozygous patients with no loss at *D11S35* was 51.4 fmol/mg, with a median of 21 fmol/mg (range, 1–187 fmol/mg). For *D11S935* the median PR of the LOH group was 8 fmol/mg (range, 2–130 fmol/mg), for the no-LOH group it was 7.5 fmol/mg (range, 1–1144 fmol/mg). For NM23 the median for the LOH group was 6 fmol/mg (range, 1–187 fmol/mg), and for the no-LOH group 10.5 fmol/mg (3–1144 fmol/mg). Plotting tumoral PR concentration against *D11S35* allele loss status as a log scatter plot (Fig. 2) shows the association of low/absent tumoral PR with *D11S35* LOH, but this clear difference is not apparent for the other two markers.

The difference between the medians in the *D11S35* LOH/no-LOH groups was statistically significant with the Mann-Whitney *U* test ($P = 0.014$). These analyses were repeated for a chromosome 11 short arm locus, *D11S935* at 11p13; and for NM23, a chromosome 17 long arm locus (*17q22*). Although large, apparent differences were also noted (see above) for the LOH and heterozygous/no loss groups at these two loci, no

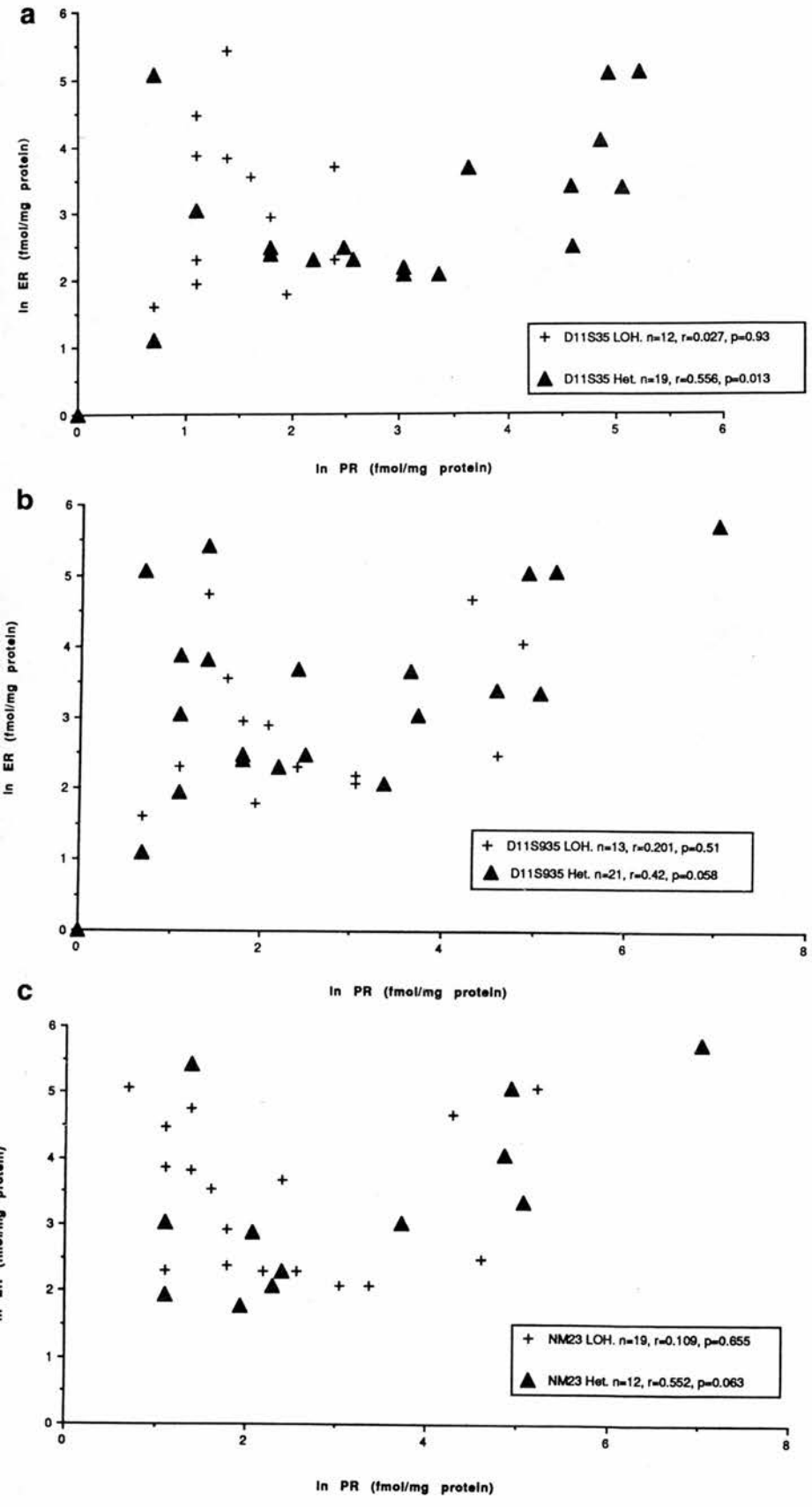


*Fig. 3* Scatter plot of natural log-transformed (*ln*) ER and PR values comparing tumors with allele loss (LOH) and tumors that were heterozygous without LOH (*Het*). Data presented: *a*, *D11S35* (11q22); *b*, *D11S935* (11p13); and *c*, NM23.

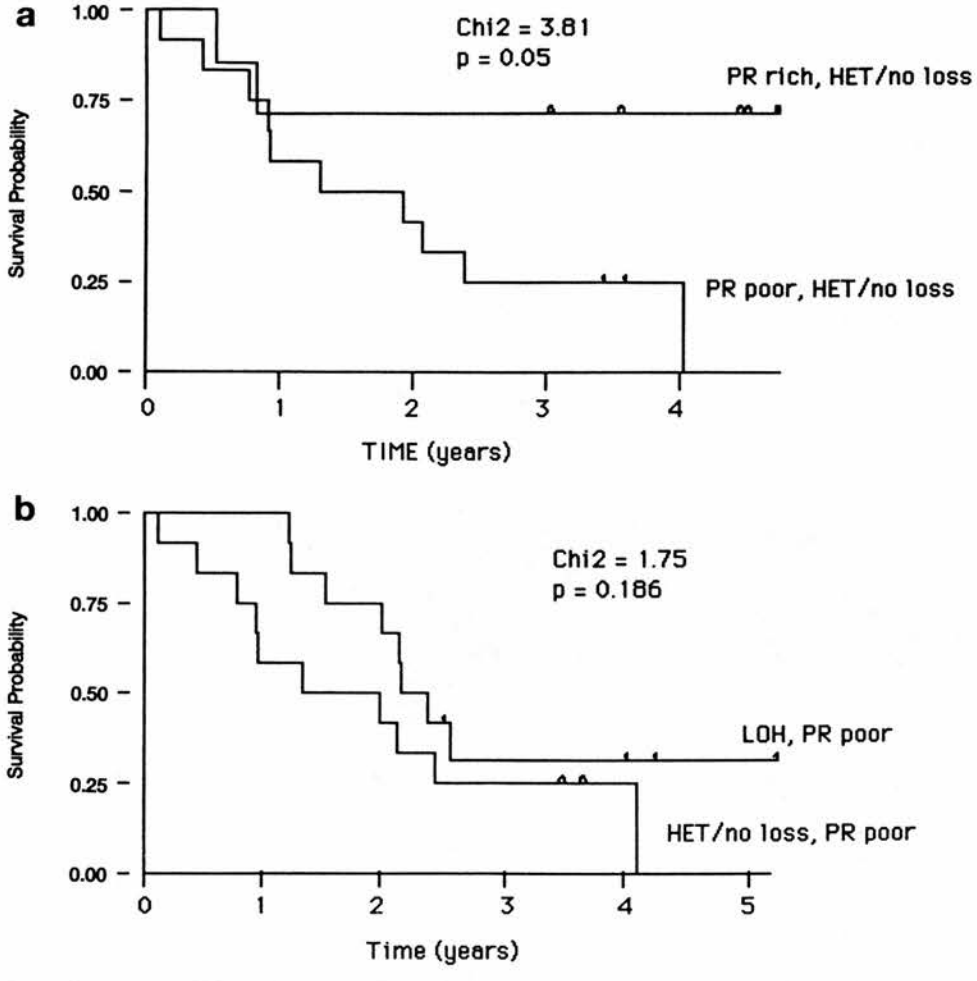


*Fig. 4* Kaplan-Meier survival curves with log rank analysis for *D11S35* heterozygous, no loss (*HET*/no loss) ovarian cancer patients subdivided by PR content (*a*) and PR-poor patients subdivided by *D11S35* allele loss status (*b*). *Vertical lines*, censored patients.

statistically significant differences of mean and median PR concentration by allele loss status were recorded (Table 2 and Fig. 2).

**Relationship between ER and PR.** The whole *D11S35* informative group was analyzed for linear correlation between tumoral ER and PR content by Spearman's rank correlation. There was no significant correlation for the whole group. However, when patients with *D11S35* LOH were removed from the analysis, there was a linear correlation between tumoral PR and ER content (Spearman's rank correlation, $P = 0.013$). Fig. 3*a* shows a scatter plot of *D11S35* heterozygous/no loss group and LOH group in terms of their ER *versus* matched PR contents. The analysis was repeated for *D11S935* (Fig. 3*b*) and NM23 (Fig. 3*c*). Although there was no significant correlation between ER and PR content in either the heterozygous/no loss group or the LOH group for either of these latter markers, it is interesting that both had trends to significance in their respective heterozygous/no loss group, but these trends were not different between the *D11S935* marker on 11p ($P = 0.058$) and NM23 on 17q ($P = 0.063$).

**Survival Analysis.** Kaplan-Meier/log rank survival analysis for ER-rich *versus* ER-poor patients showed no significant difference between the two groups (data not shown). PR-rich *versus* PR-poor showed a nonsignificant trend in favor of the PR-rich patients ($P = 0.08$, data not shown). Comparing patients with tumor *D11S35* LOH *versus* patients with *D11S35* heterozygous/no loss tumors also showed no difference (data not shown). However, analysis of patients with *D11S35* heterozygous/no loss tumors only showed a survival advantage for patients with PR-rich ($\geq$30 fmol/mg) tumors compared to those with PR-poor ($<$30 fmol/mg) tumors ($P = 0.05$, Fig. 4*a*) and PR-poor patients did equally as badly whether they had LOH at *D11S35* or not (Fig. 4*b*).

*D11S35* heterozygous/no loss patients with ER-rich tumors ($\geq$30 fmol/mg) had a better outcome compared to those with ER-poor ($<$30 fmol/mg) tumors ($P = 0.025$; Fig. 5*a*).

Patients who had informative ER-rich ($\geq$30 fmol/mg) tumors were then plotted according to whether they had LOH at *D11S35* or not (Fig. 5*b*), and a significant ($P = 0.014$) survival

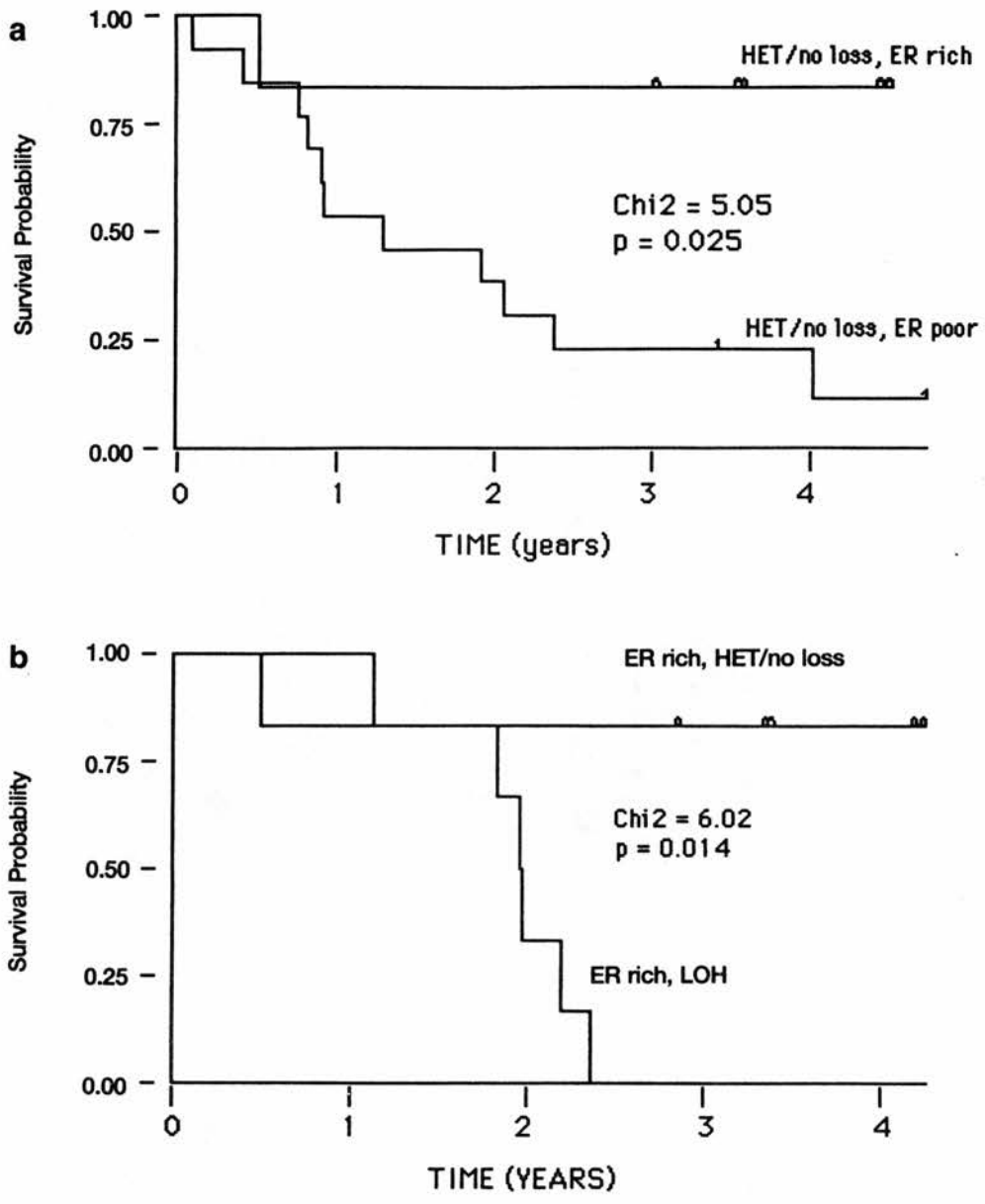


*Fig. 5* Kaplan-Meier survival curves with log rank analysis for *D11S35* heterozygous, no loss (*HET*/no loss) ovarian cancer patients subdivided by ER content (*a*) and ER-rich ovarian cancer patients subdivided by allele loss status at *D11S35* (*b*). *Vertical lines*, censored patients.

advantage in favor of those with no LOH at *D11S35* was observed (although this subgroup comprised only 12 patients).

## Discussion

This report demonstrates that allele loss close to the *PR* gene on chromosome 11q22 is associated with low tumoral PR content, suggesting the possibility that genomic structural disruption including or flanking the *PR* gene may have a significant role in the dysregulation of the PR content of at least some ovarian cancers.

That allele loss at the *PR* gene locus may genuinely disrupt PR expression is also suggested by the observation that when tumors with LOH at the *PR* gene locus are excluded from the informative sample, a significant correlation between tumoral ER and PR content is revealed, indicating that in many of these heterozygous tumors, the regulatory link between estradiol, ER, and PR is intact, and that the converse is true in those with LOH at the *PR* gene locus. Although trends also are observed for distantly physically linked (*D11S935*; 11p) and unlinked (NM23; 17q) markers, they do not reach conventional significance levels, nor are the trends substantially different from each other. (One might have expected that substantial deletions involving 11p as well as 11q, resulting in co-loss of the chromosome 11 markers, might be responsible for a higher level of borderline significance with this distant but physically linked marker than for an unlinked marker.) The fact that there are

nonsignificant trends is consistent with the likelihood that *PR* gene disruption will not be the only mechanism of tumoral PR down-regulation.

Steroid receptor negativity in the literature is variably and arbitrarily defined: in the case of ER from as low as 1–5 fmol/mg protein (indicating the background level of EIA) to between 15 and 20 fmol/mg (indicating the clinical levels below which hormonal therapeutic manipulations are unlikely to yield benefit). Interestingly, all tumors with LOH at *D11S35* had PR of 11 fmol/mg or less.

Not all previous literature is supportive of our observations. Fuqua *et al.* (22) investigated the status of RFLPs within the *PR* gene in a large series of breast tumors and found no correlation with PR expression. Additionally, none of five informative breast cancer normal/tumor pairs exhibited allele loss using one of these RFLPs, and the authors concluded that *PR* gene rearrangements were not a significant mechanism for the alteration of tumoral PR content. However, the extent of contaminating normal stroma (containing constitutive DNA) in these tumors was not discussed, and the six human breast cancer cell lines tested in the study were all homozygous at the reported RFLPs.

The observed tight link between *D11S35* LOH and loss of PR expression may be due to variations on the classical Knudson two-hit model (43). Loss of imprinting (for which there is some evidence on 11q, although not in ovarian cancer; Ref. 44) of one allele in association with large deletions of the other may occur in concert and may affect regulatory rather than structural regions, perhaps explaining the negative findings of Fuqua *et al.* (22).

Clearly, analysis of the *PR* gene locus and RNA species in these tumors is required to demonstrate the nature of these abnormalities, since the Kd68 anti-PR antibody may not detect truncated forms of the PR protein.

Analogous work with the ER in breast cancer has demonstrated an ER variant lacking exon 5 of the hormone-binding domain in ER-negative/PR-positive breast cancers that constitutively activates the ER response (45). A truncated ER species which inhibits the binding of ER to its response element has been identified in ER-positive/PR-negative breast tumors (46), and an ER variant co-species lacking exon 3 of the DNA-binding domain was identified from ER-positive tumors which was unable to function as a transcriptional inducer (47).

Kaplan-Meier survival analysis in the small subgroups of our series must be treated with caution given the sample sizes. Nevertheless, it is interesting that patients with ER-rich tumors and allele loss close to the *PR* gene had particularly poor survival; all six patients having died within 2½ years, whereas those with ER-rich tumors that retain heterozygosity at the *PR* gene locus have particularly good survival, with five of the six patients alive beyond 3 years. Clearly, such small numbers inevitably introduce bias, and this observation should perhaps stimulate a prospective analysis in a larger series of patients.

Our finding that none of the endometrioid tumors had LOH close to the *PR* gene is intriguing, and consistent with the many reports that this histological subtype is associated with higher tumoral PR content (9) and the finding in one study that endometrioid tumors can have a 54% response rate to progestin therapy (42).

The analysis of PR content in relation to survival may well be complicated by the presence of LOH in association with abnormal PR protein, which is undetected by antibodies. Removing tumors with LOH from our series and examining those that had retained heterozygosity resulted in the survival patterns that would have been expected; patients with ER-rich and PR-rich tumors exhibiting a survival advantage (Figs. 4 and 5).

We suggest that structural alteration at the *PR* locus on chromosome 11q22 may represent a significant mechanism for the dysregulation of tumoral PR content. Further analysis of the *PR* gene structure, expression, and associated clinical correlations in these tumors to determine the mechanism(s) involved will be important and is currently under way in our laboratory.

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## References

1. Gardner, W. U. Further studies on experimental ovarian tumorigenesis. Proc. Am. Assoc. Cancer Res., *2:* 300, 1958.

2. Jabara, A. G. Induction of canine ovarian tumors by diethylstilbestrol and progesterone. Aust. J. Exp. Biol., *40:* 139–152, 1962.

3. Fathalla, M. F. Incessant ovulation—a factor in ovarian neoplasia? Lancet, *2:* 163, 1971.

4. Casagrande, J. T., Louie, E. W., Pike, M. C., Roy, S., Ross, R. K., and Henderson, B. E. "Incessant ovulation" and ovarian cancer. Lancet, *2:* 170–173, 1979.

5. Cramer, D. W., Hutchison, G. B., Welch, W. R., Scully, R. E., and Ryan, K. J. Determinants of ovarian cancer risk. I. Reproductive experiences and family history. J. Natl. Cancer Inst., *71:* 711–716, 1983.

6. Weiss, N. S., Lyon, J. L., Liff, J. M., Vollmer, W. M., and Daling, J. R. Incidence of ovarian cancer in relation to the use of oral contraceptives. Int. J. Cancer, *28:* 669–671, 1981.

7. Cramer, D. W., Hutchison, G. B., Welch, W. R., Scully, R. E., and Knapp, R. C. Factors affecting the association of oral contraceptives and ovarian cancer. N. Engl. J. Med., *307:* 1047–1051, 1982.

8. Cramer, D. W., and Welch, W. R. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J. Natl. Cancer Inst., *71:* 717–721, 1983.

9. Slotman, B. J., and Rao, B. R. Ovarian cancer (Review). Anticancer Res., *8:* 417–434, 1988.

10. Bonte, J. Developments in endocrine related therapy of endometrial and ovarian cancer. Rev. Endocrinol. Rel. Cancer, *3:* 11, 1979.

11. O'Malley, B. W. The steroid receptor superfamily: More excitement predicted for the future. Mol. Endocrinol., *4:* 363–369, 1990.

12. Horwitz, K. B., and McGuire, W. L. Estrogen control of progesterone receptor in human breast cancer: correlation with nuclear processing of estrogen receptor. J. Biol. Chem., *253:* 2223–2228, 1978.

13. Langdon, S. P., Hirst, G. L., Miller, E. P., Hawkins, R. A., Tesdale, A. L., Smyth, J. F., and Miller, W. R. The regulation of growth and protein expression by estrogen *in vitro*: a study of 8 human ovarian carcinoma cell lines. J. Steroid Biochem. Mol. Biol., *50:* 131–155, 1994.

14. Friedman, M., Lagios, M., Markowwitz, A., Jones, H., Resser, K., and Hoffman, P. Estradiol (ER) and progesterone receptors (PR) in ovarian cancer—clinical and pathological correlation. Clin. Res., *27:* 385A, 1979.

15. Clark, G. M., McGuire, W. L., Hubay, C. A., Pearson, O. H., and Marshall, J. S. Progesterone receptors as a prognostic factor in stage II breast cancer. N. Engl. J. Med., *309:* 1343–1347, 1983.

16. Creasman, W. T., Sasso, R. A., Weed, J. C., Jr., and McCarty, K. S., Jr. Ovarian carcinoma: histologic and clinical correlation of cytoplasmic

estrogen and progesterone binding. Gynecol. Oncol., *12:* 319–327, 1981.

17. Iversen, O. E., Skaarland, E., and Utaaker, E. Steroid receptor content in human ovarian tumors: survival of patients with ovarian carcinoma related to steroid receptor content. Gynecol. Oncol., *23:* 65–76, 1986.

18. Rousseau-Merck, M. F., Misrahi, M., Loosfelt, H., Milgrom, E., and Berger, R. Localisation of the human progesterone receptor gene to chromosome 11q22. Hum. Genet., *77:* 280–282, 1987.

19. Law, M. L., Kao, F. T., Wei, Q., Hartz, J. A., Greene, G. L., Zarucki-Schulz, T., Conneely, O. M., Jones, C., Puck, T. T., O'Malley, B. W., and Horwitz, K. B. The progesterone receptor maps to human chromosome band 11q13, the site of the mammary oncogene int-2. Proc. Natl. Acad. Sci. USA, *84:* 2877–2881, 1987.

20. Ali, I. U., Lidereau, R., Theillet, C., and Callahan, R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science (Washington DC), *238:* 185–188, 1987.

21. Trent, J. M. Cytogenetic and molecular biological alterations in human breast cancer: a review. Breast Cancer Res. Treat., *5:* 221–229, 1985.

22. Fuqua, S. A. W., Hill, S. M., Chamness, G. C., Benedix, M. G., Greene, G. L., O'Malley, B. W., and McGuire, W. L. Progesterone receptor gene restriction fragment length polymorphisms in human breast tumors. J. Natl. Cancer Inst., *83:* 1157–1160, 1991.

23. Stickland, J. E., Tomlinson, I. P. M., Lee, A. S. G., Evans, M. F., and McGee, J. O. Allelic loss on chromosome 11q is a frequent event in breast cancer. Br. J. Cancer, *66* (Suppl. 17)*:* 3, 1992.

24. Hampton, G. M., Mannermaa, A., Winquist, R., Alavaikko, M., Blanco, G., Taskinen, P. J., Kiviniemi, H., Newsham, I., Cavenee, W. K., and Evans, G. A. Loss of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. Cancer Res., *54:* 4586–4589, 1994.

25. Carter, S. L., Negrini, M., Baffa, R., Gillum, D. R., Rosenberg, A. L., Schwartz, G. F., and Croce, C. M. Loss of heterozygosity at 11q22-q23 in breast cancer. Cancer Res., *54:* 6270–6274, 1994.

26. Magdelenat, H., Gerbault-Seureau, M., and Dutrillaux, B. Relationship between loss of estrogen and progesterone receptor expression and of 6q and 11q chromosome arms in breast cancer. Int. J. Cancer, *57:* 63–66, 1994.

27. Jenkins, R. B., Bartelt, D., Stalboerger, P., Persons, D., Dahl, R. J., Podratz, K., Keeney, G., and Hartmann, L. Cytogenetic studies of epithelial ovarian-carcinoma. Cancer Genet. Cytogenet., *71:* 76–86, 1993.

28. Foulkes, W. D., Campbell, I. G., Stamp, G., and Trowsdale, J. Loss of heterozygosity and amplification on chromosome-11q in human ovarian-cancer. Br. J. Cancer, *67:* 268–273, 1993.

29. Gabra, H., Taylor, L., Cohen, B. B., Lessels, A., Eccles, D. M., Leonard, R. C. F., Smyth, J. F., and Steel, C. M. Chromosome 11 allele imbalance and clinicopathological correlates in ovarian tumours. Br. J. Cancer, *72:* 367–375, 1995.

30. Litt, M., Sharma, V., and Luty, J. Dinucleotide repeat polymorphism at the *D11S35* locus. Nucleic Acids Res., *18:* 5921, 1990.

31. Litt, M., Kramer, P., Hauge, X. Y., Weber, J. L., Wang, Z., Wilkie, P. J., Holt, M. S., Mishra, S., Doniskeller, H., and Warnich, L. A microsatellite-based index map of human chromosome-11. Hum. Mol. Genet., *2:* 909–913, 1993.

32. James, M. R., Richard, C. W., III, Schott, J-J., Youstry, C., Clark, K., Bell, J., Terwilliger, J. D., Hazan, J., Dubay, C., Vignal, A., Agrapart, M., Imai, T., Nakamura, Y., Polymeropoulos, M., Weissenbach, J., Cox, D. R., and Lathrop, G. M. A radiation hybrid map of 506 STS markers spanning human chromosome 11. Nat. Genet., *8:* 70–76, 1994.

33. Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., and Lathrop, M. A second-generation linkage map of the human genome. Nature (Lond.), *359:* 794–801, 1992.

34. Hall, J. M., Friedman, L., Guenther, C., Lee, M. K., Weber, J. L., Black, D. M., and King, M-C. Closing in on a breast cancer gene on chromosome 17q. Am. J. Hum. Genet., *50:* 1235–1342, 1992.

35. Eccles, D., Cranston, G., Steel, C. M., Nakamura, Y., and Leonard, R. C. F. Allele losses on chromosome 17 in human epithelial ovarian cancer. Oncogene, *5:* 1599–1601, 1990.

36. Cohen, B. B., Wallace, M. R., and Crichton, D. N. A comparison of procedures for analysing microsatellite repeat polymorphisms. Mol. Cell Probes, *6:* 439–442, 1992.

37. Hawkins, R. A., Black, R., Steele, R. J. C., Dixon, J. M., and Forrest, A. P. M. Oestrogen receptor concentration in primary breast cancer and axillary node metastases. Breast Cancer Res. Treat., *1:* 245–251, 1981.

38. Hawkins, R. A., Sangster, K., Tesdale, A. L., Ferguson, W. A., Krajewski, A., Levack, P. A., and Forrest, P. Experience with new assays for oestrogen receptors using monoclonal antibodies. Biochem. Soc. Trans., *15:* 949–950, 1987.

39. Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem., *72:* 248–256, 1976.

40. Berger, U., Wilson, P., Thethi, S., McClelland, R. A., Greene, G. L., and Coombes, R. C. Comparison of an immunocytochemical assay for progesterone receptor with a biochemical method of measurement and immunocytochemical examination of the relationship between progesterone and estrogen receptors. Cancer Res., *49:* 5176–5179, 1989.

41. Lazo, J. S., Schwartz, P. E., MacLusky, N. J., Labaree, D. C., and Eisenfeld, A. J. Antiproliferative effects of tamoxifen to human ovarian carcinomas *in-vitro*. Cancer Res., *44:* 2266–2271, 1984.

42. Rendina, G. M., Donadio, C., and Giovanni, M. Steroid receptors and progestinic therapy in ovarian endometrioid carcinoma. Eur. J. Gynaecol. Oncol., *3:* 241–245, 1982.

43. Knudson, A. G. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA, *68:* 820–823, 1971.

44. Mariman, E. C. M., Vanbeersum, S. E. C., Cremers, C. W. R. J., Struycken, P. M., and Ropers, H. H. Fine mapping of a putatively imprinted gene for familial nonchromaffin paragangliomas to chromosome 11q13.1— evidence for genetic-heterogeneity. Hum. Genet., *95:* 56–62, 1995.

45. Fuqua, S. A., Fitzgerald, S. D., Chamness, G. C., Tandon, A. K., McDonnell, D. P., Nawaz, Z., O'Malley, B. W., and McGuire, W. L. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res., *51:* 105–109, 1991.

46. Fuqua, S. A., Fitzgerald, S. D., Allred, D. C., Elledge, R. M., Nawaz, Z., McDonnell, D. P., O'Malley, B. W., Greene, G. L., and McGuire, W. L. Inhibition of estrogen receptor action by a naturally occuring variant in human breast tumors. Cancer Res., *52:* 483–486, 1992.

47. Fuqua, S. A., Allred, D. C., Elledge, R. M., Krieg, S. L., Benedix, M. G., Nawaz, Z., O'Malley, B. W., Greene, G. L., and McGuire, W. L. The Er-positive/PgR-negative breast cancer phenotype is not associated with mutations within the DNA binding domain. Breast Cancer Res. Treat., *26:* 191–202, 1993.

# A statistical analysis of chromosome 11 and 17 loss of heterozygosity in epithelial ovarian cancer

HANI GABRA[1], J.E. VIVIENNE WATSON[1], DIANA M. ECCLES[3], LORNA TAYLOR[2], KAREN J. TAYLOR[1], BRIAN B. COHEN[4], ROBERT C.F. LEONARD[1], DAVID J. PORTEOUS[2], JOHN F. SMYTH[1] and C. MICHAEL STEEL[4]

[1]Imperial Cancer Research Fund Medical Oncology Unit; [2]Medical Research Council Human Genetics Unit, Western General Hospital, Edinburgh; [3]Cancer Research Campaign Genetic Epidemiology Unit, University of Southampton; [4]School of Biological and Medical Sciences, University of St. Andrews, UK

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**Abstract.** Many regions of the genome exhibit loss of heterozygosity (LOH) in epithelial ovarian cancer (EOC) suggesting sites of recessive genetic elements such as tumor suppressor genes. We performed detailed LOH studies of chromosomes 17 and 11 using 24 microsatellite repeat markers in a population of 47 patients with EOC. Univariate statistical analysis revealed that significant co-losses of chromosomal loci occurred between 17p and 17q whole arms (p=0.0003), NME1 (17q21) with D11S922 (11p15.5) (p=0.0067) and D11S912 (11q24) with D11S935 (11p13) (p=0.0073). Statistical analysis of the relationship between LOH on particular chromosomal arms and clinicopathological factors revealed a significant association between serous histological subtype of ovarian adenocarcinoma and chromosome 17p (p=0.0052) and telomeric 17q (p=0.0007) LOH. An analysis of specific polymorphic chromosomal loci demonstrated that adverse survival was significantly associated with LOH at 11q24 (p=0.0067) and 17q21 (p=0.0076). There were non-significant trends suggesting a relationship between chromosome 17p LOH and poorly differentiated (p=0.025) and advanced FIGO stage (p=0.031) tumours. Considering these statistical associations, a preliminary multistep model for involvement of chromosomes 11 and 17 in ovarian neoplasia can be constructed.

## Introduction

Ovarian cancer is the commonest cause of death from gynaecological malignancy in the developed world. Its poor prognosis is primarily the result of its late presentation due to early symptoms being insidious in onset and non-specific in nature.

Cytogenetic studies have revealed that non-random chromosomal abnormalities occur frequently in ovarian cancer (1-5). Cumulative genetic lesions experienced by the ovarian surface epithelium provide a multistep pathway to ovarian neoplasia.

Inactivation of recessive genetic elements such as tumour suppressor genes represent frequent and important events en-route to frank neoplasia (6). Evidence for such inactivation events may be inferred from loss of heterozygosity (LOH) when it is demonstrated in DNA from a tumour sample compared with matched constitutive DNA (7). Frequent LOH has been demonstrated for many chromosomes in ovarian cancer. Among these are chromosomes 17 and 11. Chromosome 17 (carrying p53 on p13.3 and BRCA1 on q21.2) has been implicated in many tumour types, and is believed, in many instances to be lost in its entirety (as a whole homologue), with or without reduplication of the other chromosome in ovarian cancer (8,9). Chromosome 11p15.5 has been implicated in ovarian (10-13), breast (14,15) and other cancers. The 11p13 region exhibits lower LOH rates in ovarian cancer. It is of potential interest since it is the approximate site of wt1 and has been investigated by several groups (16,17). 11q13, the site of an 'amplicon' involved in breast cancer, exhibits less LOH in ovarian cancer (18). More recently it has been demonstrated that the 11q22-qter region exhibits high levels of LOH in melanoma (19), breast (14,15,20,21), ovarian (10,18), cervical (22) and lung (23) cancer.

In previous studies, we determined the extent of LOH in ovarian cancer using 24 microsatellite markers; 14 located on chromosome 17, and 10 located on chromosome 11. In this study we present the results of a univariate statistical analysis in 47 EOCs comparing LOH rates at these loci and their association with loss of other loci, and with prognostically important clinicopathological features of ovarian cancer.

## Materials and methods

*Clinical specimens.* Fresh primary ovarian tumour tissue from patients was transferred directly to dry ice or liquid

*Correspondence to*: Dr H. Gabra, ICRF Medical Oncology Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

*Key words:* ovarian cancer, loss of heterozygosity, chromosome 11, chromosome 17, survival, prognostic factors, microsatellite markers

Table I. Patient characteristics.

| | | | |
|---|---|---|---|
| Ovarian adenocarcinoma | | | 47 |
| Histology | Serous | | 25 |
| | Endometrioid | | 14 |
| | Mucinous | | 5 |
| | Clear cell | | 3 |
| Differentiation | Well | | 3 |
| | Moderate | | 14 |
| | Poor | | 25 |
| | Not known | | 5 |
| Stage | I/II | | 16 |
| | III/IV | | 29 |
| | Not known | | 2 |
| Surgical treatment | Completely debulked | | 32 |
| | Incompletely debulked | | 15 |
| | Not known | | 2 |
| Chemotherapy | Chlorambucil: | Adjuvant | 3 |
| | | Palliative | 6 |
| | Cis-platinum: | Adjuvant | 11 |
| | | Palliative | 8 |
| | Carboplatinum: | Palliative | 2 |
| | None | | 13 |

nitrogen and stored at -70°C until processing. Heparinised blood was obtained postoperatively. FIGO staging and histopathology (including differentiation) were determined and reviewed in a standard fashion at a multidisciplinary combined gynaecological oncology clinic. Treatment was planned and delivered in accordance with established protocols implemented by the multidisciplinary clinic at the time, comprising the best possible surgical debulking followed by adjuvant/palliative chemotherapy. Minimum follow-up is 33 months, and all deaths that have occurred have been due to ovarian cancer. Patient characteristics are outlined in Table I.

*DNA extraction*. DNA from fresh frozen tissue was extracted by a standard technique as previously described (24).

*Oligonucleotide primers*. Chromosome 11 oligonucleotide primers were selected on the basis of recently generated microsatellite index maps (25-28) for locus, informativeness and spacing as previously described. Chromosome 17 microsatellite primers were derived from the human genome mapping project. D17S250, THRA1, NME1 and GH1 are listed in a recent mapping compendium (29). Other sources were used to derive the remaining microsatellites D17S579 (30), D17S293 (30) and D17S588 (31).

The approximate positions of the chromosome 11 and 17 polymorphic markers used in this study are shown in Fig. 1.

*Polymerase chain reaction and polymorphic microsatellite detection*. PCR was performed in a reaction volume of 50 µl. 100 ng DNA was amplified using reaction conditions specified

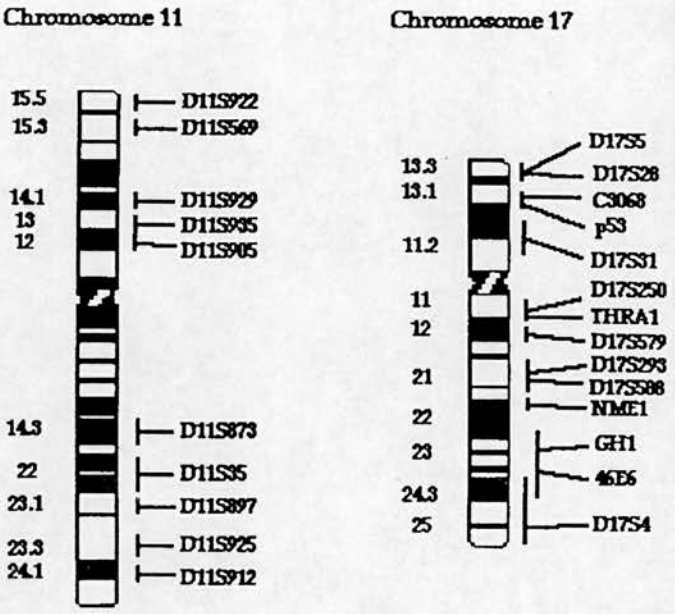


Figure 1. Chromosomal location of polymorphic loci used in this analysis.

in the original papers. PCR reaction product was loaded onto 6% or 9% denaturing polyacrylamide gel, separated by electrophoresis, passively transferred to Hybond nylon and probed with $^{32}P$ end-labelled (using polynucleotide kinase) poly(CA) probe as previously described (32). Two observers visually analysed the autoradiographs and recorded LOH when there was clear reduction in intensity of one allele in tumour DNA.

*Southern blot analysis*. Southern blot analysis was performed for the chromosome 17p markers and D17S4 (THH59) as previously described (24,33) (Table II). D17S5, D17S28, C3068, D17S31 and D17S4 (34) were DNA probes used for Southern analysis and had generally lower heterozygosity rates than the CA repeat PCR probes used for the analysis. The approximate positions of the chromosome 17 polymorphic markers used in this study are shown in Fig. 1.

*Statistical analysis*. The two-tailed Fisher's exact test was used to determine if there were significant associations between LOH at different loci on chromosome 11 and 17. The same univariate analysis was applied to examine relationships between LOH at any locus and FIGO stage, differentiation grade, histological subtype or survival. Since numerous Fisher's analyses were carried out, the cut-off for significance was set at $p \leq 0.01$, but we have included trends towards significance in the region of $0.07 > p > 0.01$ where they have supported or suggested biological hypotheses. Kaplan-Meier/log-rank analysis was performed to determine any relationship between LOH and survival. Multivariate analysis was not attempted since the small initial population precluded further reduction in number by sub-grouping for this type of analysis.

## Results

*LOH at chromosomes 11 and 17*. Table II shows the frequency of LOH at the loci tested and their locations. Chromosome 17

Table II. Locus name, position LOH rate for markers used in the analysis.

| | Chromosome 17 | | | | | Chromosome 11 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Marker | Location | LOH | Informative | %LOH | Marker | Location | LOH | Informative | %LOH |
| p arm | D17S5 | 17p13.3 | 9 | 17 | 53 | D11S922 | 11p15.5 | 16 | 36 | 44 |
| | D17S28 | 17p13.3 | 9 | 19 | 47 | D11S569 | 11p15.3 | 14 | 30 | 47 |
| | C3068 | 17p13.3-p13.1 | 9 | 19 | 47 | D11S929 | 11p14.1 | 9 | 38 | 24 |
| | p53 | 17p13.1 | 11 | 20 | 55 | D11S935 | 11p13 | 13 | 39 | 33 |
| | D17S31 | 17p13.1-p11.2 | 5 | 13 | 38 | D11S905 | 11p13-p12 | 15 | 33 | 45 |
| q arm | D17S250 | 17q11-q12 | 15 | 29 | 52 | D11S873 | 11q14.3 | 9 | 22 | 41 |
| | THRA1 | 17q11.2-q12 | 17 | 28 | 61 | D11S35 | 11q22 | 14 | 39 | 36 |
| | D17S579 | 17q12-q21 | 18 | 35 | 51 | D11S897 | 11q23.1 | 9 | 32 | 28 |
| | D17S293 | 17q21 | 14 | 32 | 44 | D11S925 | 11q23.3 | 18 | 33 | 54 |
| | D17S588 | 17q21 | 18 | 33 | 55 | D11S912 | 11q24.1 | 18 | 37 | 49 |
| | NME 1 | 17q21-q22 | 25 | 38 | 66 | | | | | |
| | GH1 | 17q22-q24 | 9 | 33 | 27 | | | | | |
| | 46 E 6 | 17q22-q24.3 | 23 | 41 | 56 | | | | | |
| | D17S4 | 17q23-q25.3 | 13 | 19 | 68 | | | | | |

Table III. Statistical associations between chromosome 11 and 17 arm LOH.

| Whole arm Co-losses | p-value | n |
|---|---|---|
| 17p vs 17q | 0.0003 | 37 |
| 17p vs 11p | 1 | 34 |
| 17q vs 11q | 0.0351 | 41 |
| 17p vs 11q | 0.119 | 32 |
| 17q vs 11p | 0.624 | 43 |
| 11p vs 11q | 0.651 | 41 |
| 17q vs 11q23-qter | 0.0175 | 40 |

Table IV. Statistical associations between LOH at defined polymorphic loci.

| Co-losses | Location | Number informative | p-value |
|---|---|---|---|
| NME1, D11S922 | 17q21-22, 11p15.5 | 30 | 0.0067 |
| D11S912, D11S935 | 11q24.1, 11p13 | 32 | 0.0073 |
| p53, D17S4 | 17p13.1, 17q23-25 | 12 | 0.018 |
| D11S912, D11S929 | 11q24.1, 11p14.1 | 34 | 0.019 |
| THRA-1, D11S35 | 17q11-12, 11q22 | 18 | 0.049 |

LOH was generally higher than chromosome 11 LOH. Rates were lowest at GH1 on chromosome 17q and D11S929 on chromosome 11p. The individual LOH rates for chromosome 11 (10) in all tumours and 17p (24,33) in most tumours have been reported previously. The 17q data, however, are previously unreported.

*Correlations between LOH at different polymorphic microsatellite loci.* The polymorphic marker LOH data were initially used for a 'whole arm' analysis to test if some of the chromosome arms were lost together more frequently than predicted by chance (Table III). In this analysis, co-loss of 17p and 17q was noted with a high degree of significance (p=0.0003). Co-loss of 17q and 11q also showed a trend to significance (p=0.035) and this trend became more significant when the telomeric half of 11q was considered (p=0.0175).

Individual loci were then examined for evidence of significant co-ordinate loss (Table IV). In this analysis, adjacent loci demonstrating significant co-loss were ignored since this might have been due to contiguous deletions. Within the confines of this assumption, NME1 (17q21) and D11S922 (11p15.5) were lost together very significantly (p=0.0067) as were D11S912 (11q24.1) and D11S935 (11p13) (p=0.0073). Co-ordinate losses of p53/D17S4 (17p13/17q25) and D11S912/D11S929 (11q24/11p14) both occurred at borderline significance. The only individual loci in this study demonstrating 17q/11q co-loss were Thra1 and D11S35 at borderline significance (p=0.049).

*LOH and correlation with clinicopathological factors.* Fisher's test was then used to examine the relationship between whole arm or individual locus LOH with clinicopathological prognostic factors.

The only significant whole arm LOH association (Table V) was between 17p LOH and serous type histology (p=0.0052). There was also a trend towards significance for 17p LOH with poorly differentiated tumours (p=0.025) and advanced FIGO stage (p=0.031), but no association with survival (p=0.171). The only other 'whole arm' trend to significance was found for 11q LOH with adverse survival (p=0.031).

Table V. Statistical association between chromosome arm loss and clinicopathological features of ovarian cancer.

| Prognostic parameter associated | Chromosome arm associated | Number informative | Two-tailed p-value |
|---|---|---|---|
| Serous histology | 17p | 37 | 0.0052 |
| | 17q | 47 | 0.123 |
| | 11p | 43 | 1 |
| | 11q | 41 | 0.306 |
| Poorly differentiated tumour | 17p | 34 | 0.025 |
| | 17q | 42 | 0.099 |
| | 11p | 38 | 0.709 |
| | 11q | 36 | 0.483 |
| Advanced FIGO stage | 17p | 35 | 0.031 |
| | 17q | 44 | 0.434 |
| | 11p | 40 | 0.689 |
| | 11q | 38 | 0.061 |
| Adverse survival outcome | 17p | 36 | 0.171 |
| | 17q | 46 | 0.117 |
| | 11p | 42 | 0.708 |
| | 11q | 40 | 0.031 |

Individual loci were then examined for an association between LOH and prognostic clinicopathological factors (Table VI). In this part of the analysis, individual 17p locus LOH always occurred in concert, and so 17p loss was considered as a single entity. D17S4 (17q25) LOH was highly significantly associated with serous histology (p=0.0007) (loss of D17S4 co-incided significantly with loss of 17p, associated with serous histology). D17S588 LOH (17q21) was significantly associated with advanced FIGO stage (p=0.0075). LOH at two loci were significantly associated with adverse survival; D11S912 (11q24; p=0.0067) and Thra1 (17q11-12; p=0.0076). Other trends towards significance are shown in Table VI including two associations of LOH which deserve further comment: D11S35 LOH was associated with non-endometrioid histology (p=0.04), interesting because of the proximity of the progesterone receptor to this locus (35); and D11S873 LOH associated with moderate/well differentiated tumours at borderline significance (p=0.07).

*Survival analysis.* Kaplan-Meier/log-rank analysis confirmed the Fisher's exact test findings for D11S912 [previously described (10)] and Thra1, with a significant actuarial survival disadvantage for those who had LOH at either of those two loci (Fig. 2).

## Discussion

This analysis demonstrates that chromosome 17 and 11 LOH affecting individual polymorphic loci and whole chromosome arms show significant concordant events and associations with prognostically important clinicopathological features of ovarian cancer. The data presented in this study do not allow interpretation of the prognostic independence of these markers, as only associations can be reported with this type of univariate analysis.

Most striking among the 'whole arm' co-loss events is the association between 17p and 17q LOH. This has been described previously in allelotype analyses using fewer

Table VI. Statistical association between defined polymorphic loci and clinicopathological features of ovarian cancer.

| Prognostic parameter associated with LOH | Microsatellite marker associated | Location | Number informative | Significance of statistical association |
|---|---|---|---|---|
| Histology: | | | | |
| serous | D17S4 | 17q23-25.3 | 18 | 0.0007 |
| | 17p | | 37 | 0.0052 |
| | D17S588 | 17q21 | 33 | 0.038 |
| non-endometrioid | D11S35 | 11q22 | 27 | 0.04 |
| Differentiation: | | | | |
| poor | D17S4 | 17q23-25.3 | 18 | 0.022 |
| | D17S579 | 17q12-21 | 31 | 0.023 |
| | 17p | | 34 | 0.025 |
| | THRA-1 | 17q11.2-12 | 24 | 0.03 |
| moderate/well | D11S873 | 11q14.3 | 19 | 0.07 |
| Advanced FIGO stage | D17S588 | 17q21 | 31 | 0.0075 |
| | D11S912 | 11q24.1 | 35 | 0.035 |
| Adverse survival outcome | D11S912 | 11q24.1 | 36 | 0.0067 |
| | THRA-1 | 17q11.2-12 | 27 | 0.0076 |
| | NME1 | 17q21-22 | 37 | 0.047 |

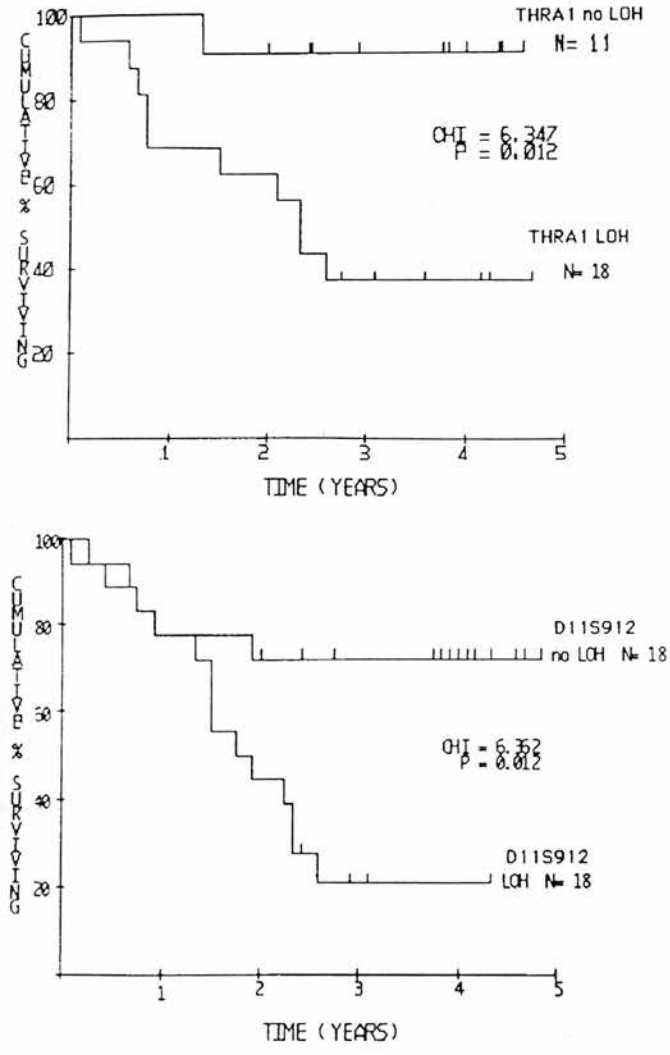


Figure 2. Kaplan-Meier/log-rank survival analyses comparing patients with or without loss of heterozygosity (LOH) at THRA-1 (upper graph); and patients with or without LOH at D11S912 (lower graph).

polymorphic markers than this study (8,9); and our findings are also highly supportive of the hypothesis that chromosome 17 whole homologue loss is a very common event in ovarian cancer. In contrast, there is no association between 11p and 11q LOH in the same patient population, demonstrating that although LOH on chromosome 11 is a frequent event, it is unlikely to be explained by whole homologue loss (by mechanisms such as non-disjunction postulated to be important in chromosome 17). There is also an association between telomeric 11q LOH and 17q LOH, which has also been observed for breast cancer (36). The significance of this observation is not clear, although both these regions contain polymorphic markers where LOH is associated with adverse survival (see below).

Significant associations of LOH between individual polymorphic markers were also noted in this study, and for some of these, plausible candidates or biological hypotheses exist. It has been recognised in a substantial body of work that a putative tumour suppressor gene resides at chromosome 11p15.5 in ovarian (10-13) and other (15,37-39) cancers. Additionally, it is recognised that the nm23 gene may play a role as a progression suppressor in ovarian cancer (40). It is therefore of interest that co-loss of D11S922 at 11p15.5 and NME1 at 17q21 (site of nm23) should occur so significantly. Similarly LOH of D11S912 [associated with adverse survival in ovarian and more recently, breast cancer (41)] was found to be significantly associated with D11S935 at 11p13, known to be the approximate site of the Wilms' tumour gene (wt1). Significant rates of LOH at 11p13 have been noted in previous analyses, although SSCP and DNA sequencing in two studies failed to demonstrate any wt1 mutation in these tumours with 11p13 LOH (16,17). Several observations were made in this analysis with regard to the association of LOH with prognostically important clinicopathological factors in ovarian cancer. Whole arm loss was significantly associated with clinicopathological factors only for chromosome 17p. This is perhaps not surprising when one considers that of the four chromosome arms in this study, only 17p appeared to be lost uniformally as a whole arm, i.e. all 17p polymorphic markers

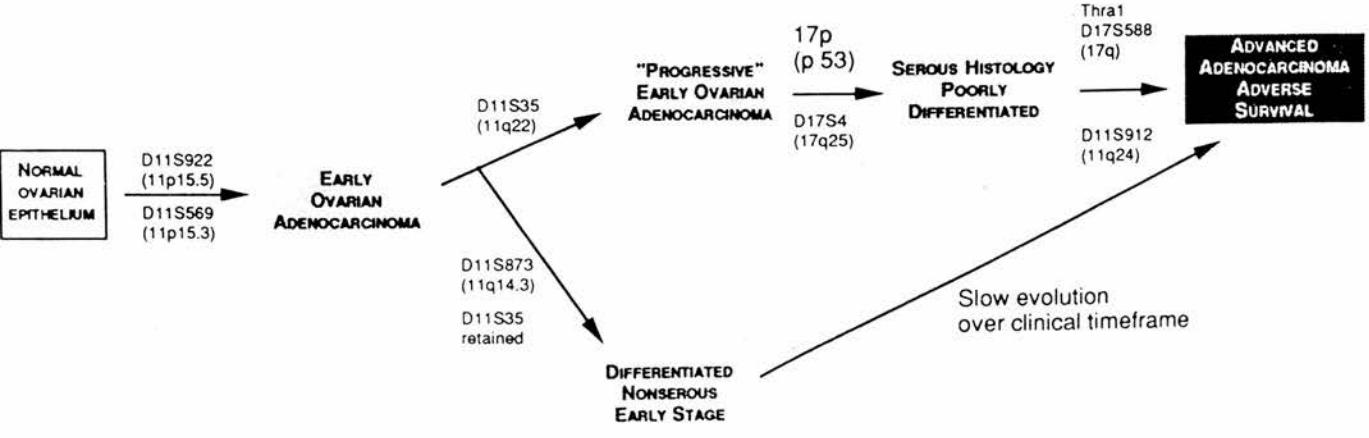


Figure 3. Integration of statistical associations of regional loss of heterozygosity with clinicopathological features on chromosomes 11 and 17 into a preliminary multistep model of ovarian carcinogenesis.

underwent LOH together. This may be a reflection of the importance of the p53 gene and its role in neoplasia. The most significant association noted was of 17p LOH with serous ovarian adenocarcinoma. The observation of p53 mutation in serous ovarian cancer is well described in several previous studies (42-45). 17p LOH is also associated with poor differentiation of the primary tumour and advanced stage at presentation, although these associations are at borderline significance. However, there was no association of 17p LOH with adverse survival. It is of interest that several extensive studies have reported no relationship between p53 abnormality and survival in ovarian cancer (43,46,47). The only other notable 'whole arm' observation was an association of borderline significance between 11q whole arm loss and adverse survival.

An analysis of LOH at individual polymorphic loci with prognostic clinicopathological features revealed several significant associations. A marker at 17q25 (D17S4) was noted to be significantly associated with serous histology, which we have previously reported (33). A marker at 17q21 was significantly associated with advanced FIGO stage (D17S588). Finally, two markers, D11S912 at 11q24 (10) and THRA1 at 17q21 were found to be associated significantly with adverse survival, suggesting the potential presence of late-acting progression-suppressors involved in ovarian cancer. If one considers that polymorphic markers undergoing frequent LOH, where no significant difference is noted between favourable and adverse clinicopathological features, may represent early events in ovarian carcinogenesis; while polymorphic markers segregating with adverse clinicopathological phenotypes may be intermediate or late acting events (depending on phenotype), then one can begin to construct a crude picture of the possible involvement of chromosomes 17 and 11 in a multistep model of ovarian neoplasia similar to one that we have previously postulated for chromosome 11 alone (10) (Fig. 3). In this model, 17p (p53) LOH represents an intermediate or early event; 17q and telomeric 11q LOH represent late events. Although the evidence is inconclusive, we have included D11S35 LOH as an early event in neoplasia, perhaps involving the progesterone receptor (35), and D11S873 LOH as an early event in generating a genetically distinct, prognostically advantageous, subgroup which presents as the relatively rare early stage form of ovarian adenocarcinoma.

It is clear that this univariate analysis is a crude tool with limits to the extent of inferences that one can draw about ovarian neoplasia. However, it does allow some general observations to be made regarding comparisons of subchromosomal abnormalities in a small but well-defined cohort of patients with ovarian cancer. It is to be hoped that refinement of these regions will provide further mechanistic answers to the observed associations as positional cloning efforts ultimately yield genes involved in these processes.

## References


1. Jenkins RB, Bartelt D, Stalboerger P, Persons D, Dahl RJ, Podratz K, Keeney G and Hartmann L: Cytogenetic studies of epithelial ovarian-carcinoma. Cancer Genet Cytogenet 71: 76-86, 1993.
2. Kiechleschwarz M, Bauknecht T, Schmidt J, Walz L and Pfleiderer A: Recurrent cytogenetic aberrations in human ovarian carcinomas. Cancer Detection Prev 19: 234-243, 1995.
3. Pejovic T, Heim S, Mandahl N, Baldetorp B, Elmfors B, Floderus UM, Furgyik S, Helm G, Himmelmann A, Willen H and Mitelman F: Chromosome-aberrations in 35 primary ovarian carcinomas. Gene Chromosome Cancer 4: 58-68, 1992.
4. Thompson FH, Emerson J, Alberts D, Liu Y, Guan XY, Burgess A, Fox S, Taetle R, Weinstein R and Makar R: Clonal chromosome-abnormalities in 54 cases of ovarian-carcinoma. Cancer Genet Cytogenet 73: 33-45, 1994.
5. Pejovic T: Genetic changes in ovarian-cancer. Ann Med 27: 73-78, 1995.
6. Knudson AG: Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 68: 820-823, 1971.
7. Ponder B: Gene losses in human tumours. Nature 335: 400-402, 1988.
8. Foulkes WD, Black DM, Stamp GWH, Solomon E and Trowsdale J: Very frequent loss of heterozygosity throughout chromosome 17 in sporadic ovarian-carcinoma. Int J Cancer 54: 220-225, 1993.
9. Osborne RJ and Leech V: Polymerase chain-reaction allelotyping of human ovarian-cancer. Br J Cancer 69: 429-438, 1994.
10. Gabra H, Taylor L, Cohen BB, Lessels A, Eccles DM, Leonard RCF, Smyth JF and Steel CM: Chromosome 11 allele imbalance and clinicopathological correlates in ovarian-tumors. Br J Cancer 72: 367-375, 1995.
11. Kiechleschwarz M, Bauknecht T, Wienker T, Walz L and Pfleiderer A: Loss of constitutional heterozygosity on chromosome 11p in human ovarian-cancer-positive correlation with grade of differentiation. Cancer 72: 2423-2432, 1993.
12. Vandamme B, Lissens W, Amfo K, Desutter P, Bourgain C, Vamos E and Degreve J: Deletion of chromosome 11p13-11p15.5 sequences in invasive human ovarian-cancer is a subclonal progression factor. Cancer Res 52: 6646-6652, 1992.
13. Viel A, Giannini F, Tumiotto L, Sopracordevole F, Visentin MC and Boiocchi M: Chromosomal localization of 2 putative 11p oncosuppressor genes involved in human ovarian-tumors. Br J Cancer 66: 1030-1036, 1992.
14. Hampton GM, Mannermaa A, Winquist R, Alavaikko M, Blanco G, Taskinen PJ, Kiviniemi H, Newsham I, Cavenee WK and Evans GA: Loss of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. Cancer Res 54: 4586-4589, 1994.
15. Negrini M, Rasio D, Hampton GM, Sabbioni S, Rattan S, Carter SL, Rosenberg AL, Schwartz GF, Shiloh Y, Cavenee WK and Croce CM: Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. Cancer Res 55: 3003-3007, 1995.
16. Bruening W, Gros P, Sato T, Stanimir J, Nakamura Y, Housman D and Pelletier J: Analysis of the 11p13 Wilms-tumor suppressor gene (wt1) in ovarian-tumors. Cancer Invest 11: 393-399, 1993.
17. Viel A, Giannini F, Capozzi E, Canzonieri V, Scarabelli C, Gloghini A and Boiocchi M: Molecular mechanisms possibly affecting wt1 function in human ovarian-tumors. Int J Cancer 57: 515-521, 1994.
18. Foulkes WD, Campbell IG, Stamp GWH and Trowsdale J: Loss of heterozygosity and amplification on chromosome 11q in human ovarian-cancer. Br J Cancer 67: 268-273, 1993.
19. Herbst RA, Larson A, Weiss J, Cavenee WK, Hampton GM and Arden KC: A defined region of loss of heterozygosity at 11q23 in cutaneous malignant melanoma. Cancer Res 55: 2494-2496, 1995.
20. Carter SL, Negrini M, Baffa R, Gillum DR, Rosenberg AL, Schwartz GF and Croce CM: Loss of heterozygosity at 11q22-q23 in breast cancer. Cancer Res 54: 6270-6274, 1994.
21. Stickland JE, Tomlinson IPM, Lee ASG, Evans MF and McGee JO: Allelic loss on chromosome 11q is a frequent event in breast cancer. Br J Cancer 66 (Suppl. XVII): 3, 1992.
22. Hampton GM, Penny LA, Baergen RN, Larson A, Brewer C, Liao S, Busby-Earle RMC, Williams AWR, Steel CM, Bird CC, Stanbridge EJ and Evans GA: Loss of heterozygosity in cervical carcinoma: subchromosomal localisation of a putative tumor-suppressor gene to chromosome 11q22-q24. Proc Natl Acad Sci USA 91: 6953-6957, 1994.
23. Rasio D, Negrini M, Maneneti G, Draggani TA and Croce CM: Loss of heterozygosity at chromosome 11q in lung adenocarcinoma: identification of three independent regions. Cancer Res 55: 3988-3991, 1995.
24. Eccles D, Cranston G, Steel CM, Nakamura Y and Leonard RCF: Allele losses on chromosome 17 in human epithelial ovarian cancer. Oncogene 5: 1599-1601, 1990.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, Bernardi G, Lathrop M and Weissenbach J: The 1993-94 Genethon human genetic linkage map. Nature Genetics 7: 246-300, 1994.
Litt M, Kramer P, Hauge XY, Weber JL, Wang Z, Wilkie PJ, Holt MS, Mishra S, Doniskeller H and Warnich L: A microsatellite-based index map of human chromosome 11. Hum Mol Genet 2: 909-913, 1993.
Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G and Lathrop M: A second-generation linkage map of the human genome. Nature 359: 794-801, 1992.
Couillin P, Leguern E, Vignal A, Fizames C, Ravise N, Delportes D, Reguigne I, Rosier M, Junien C, Vanheyningen V and Weissenbach J: Assignment of 112 microsatellite markers to 23 chromosome 11 subregions delineated by somatic hybrids-comparison with the genetic-map. Genomics 21: 379-387, 1994.
Fain PR and Solomon E: Report of the committee on the genetic constitution of chromosome 17. In: Human Gene Mapping 1994. Cuticchia AJ (ed). Johns Hopkins, Baltimore, pp647-678, 1995.
Hall JM, Friedman L, Guenther C, Lee MK, Weber JL, Black DM and King MC: Closing in on a breast cancer gene on chromosome 17q. Amer J Hum Genet 50: 1235-1242, 1992.
Jacobs IJ, Smith SA, Wiseman RW, Futreal PA, Harrington T, Osborne RJ, Leech V, Molyneux A, Berchuck A, Ponder BA, *et al:* A deletion unit on chromosome 17q in epithelial ovarian tumors distal to the familial breast/ovarian cancer locus. Cancer Res 53: 1218-1221, 1993.
Cohen BB, Wallace MR and Crichton DN: A comparison of procedures for analysing microsatellite repeat polymorphisms. Mol Cell Probe 6: 439-442, 1992.
Eccles DM, Russell S, Haites NE, Atkinson R, Bell DW, Gruber L, Hickey I, Kelly K, Kitchener H and Leonard R: Early loss of heterozygosity on 17q in ovarian-cancer. Oncogene 7: 2069-2072, 1992.
Nakamura Y, Leppert M and O'Connell P: A mapped set of DNA markers for human chromosome 17. Genomics 2: 302-309, 1988.
Gabra H, Langdon SP, Watson JEV, Hawkins RA, Cohen BB, Taylor L, Mackay J, Steel CM, Leonard RCF and Smyth JF: Loss of heterozygosity at 11q22 correlates with low progesterone receptor content in epithelial ovarian cancer. Clin Cancer Res 1: 945-953, 1995.
Thompson AM, Cohen B, Crichton D, Wallace M, McKenzie I, Steel CM: Association of 17q with 11q allele loss and with 5 year survival in human breast cancer. Breast 4: 230, 1995.
Koi M, Johnson LA, Kalikin LM, Little PFR, Nakamura Y and Feinberg AP: Tumor-cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. Science 260: 361-364, 1993.
Loh WE, Scrable HJ, Livanos E, Arboleda MJ, Cavenee WK, Oshimura M and Weissman BE: Human chromosome 11 contains 2 different growth suppressor genes for embryonal rhabdomyosarcoma. Proc Natl Acad Sci USA 89: 1755-1759, 1992.
Reeve AE, Sih SA, Raizis AM and Feinberg AP: Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumour cells. Mol Cell Biol 9: 1799-1803, 1989.
Viel A, Dallagnese L, Canzonieri V, Sopracordevole F, Capozzi E, Carbone A, Visentin MC and Boiocchi M: Suppressive role of the metastasis-related nm23-h1 gene in human ovarian carcinomas-association of high messenger-RNA expression with lack of lymph-node metastasis. Cancer Res 55: 2645-2650, 1995.
Winqvist R, Hampton GM, Mannermaa G, Blanco G, Alavaikko M, Kiviniemi H, Taskinen PJ, Evans GA, Wright FA, Newsham I and Cavenee WK: Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res 55: 2660-2664, 1995.
Eccles DM, Brett L, Lessels A, Gruber L, Lane D, Steel CM and Leonard RCF: Overexpression of the p53 protein and allele loss at 17p13 in ovarian-carcinoma. Br J Cancer 65: 40-44, 1992.
Niwa K, Itoh M, Murase T, Morishita S, Itoh N, Mori H and Tamaya T: Alteration of p53 gene in ovarian-carcinoma-clinicopathological correlation and prognostic-significance. Br J Cancer 70: 1191-1197, 1994.
Kupryjanczyk J, Thor AD, Beauchamp R, Merritt V, Edgerton SM, Bell DA and Yandell DW: P53 gene-mutations and protein accumulation in human ovarian-cancer. Proc Natl Acad Sci USA 90: 4961-4965, 1993.
Klemi PJ, Takahashi S, Joensuu H, Kiilholma P, Narimatsu E and Mori M: Immunohistochemical detection of p53 protein in borderline and malignant serous ovarian-tumors. Int J Gynecol Pathol 13: 228-233, 1994.
Kohler MF, Kerns BJM, Humphrey PA, Marks JR, Bast RC and Berchuck A: Mutation and overexpression of p53 in early-stage epithelial ovarian-cancer. Obstet Gynecol 81: 643-650, 1993.
Sheridan E, Silcocks P, Smith J, Hancock BW and Goyns MH: P53 mutation in a series of epithelial ovarian cancers from the UK, and its prognostic-significance. Eur J Cancer 11: 1701-1704, 1994.

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*Advances in Brief*

# Definition and Refinement of a Region of Loss of Heterozygosity at 11q23.3-q24.3 in Epithelial Ovarian Cancer Associated with Poor Prognosis

**Hani Gabra,[1] J. E. Vivienne Watson, Karen J. Taylor,[2] James Mackay, Robert C. F. Leonard, C. Michael Steel, David J. Porteous, and John F. Smyth**

*Imperial Cancer Research Fund Medical Oncology Unit and Medical Research Council Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU [H. G., J. E. V. W, K. T., J. M., R. C. F. L., D. J. P., J. F. S], and School of Biological and Medical Sciences, University of St. Andrews, St. Andrews, [C. M. S.], United Kingdom*

## Abstract

**Previous cytogenetic and loss of heterozygosity (LOH) data suggest that disruption of chromosome 11q23-qter occurs frequently in epithelial ovarian cancer and is associated with an adverse clinicopathological phenotype. Ten polymorphic microsatellite repeat loci were analyzed by PCR from the 11q22-q25 region between *D11S35* and *D11S968* in 40 ovarian tumors (including 31 epithelial ovarian cancers). Two distinct regions of loss were detected, suggesting possible sites for genes involved in epithelial ovarian neoplasia: a large centromeric region between *D11S35* and *D11S933* (11q22-q23.3) and a telomeric 8.5-Mb region lying between *D11S934* and *D11S1320* (11q23.3–24.3) not previously defined. LOH of the latter region but not the former one was significantly associated with poor survival, despite all tumors in this study having LOH somewhere on chromosome 11. This analysis provides a starting point for positional cloning.**

## Introduction

EOC[3] the most common cause of death from gynecological malignancy, is typically a late-presenting disease with poor prognosis. The pathophysiological pathways to this common, aggressive phenotype are determined by multiple genetic lesions accumulated by the ovarian surface epithelial cells. Inactivation of tumor suppressor genes are frequent and important components of these pathways, and regions of the genome containing such genes may be delineated by cytogenetic and LOH analyses. The list of putative tumor suppressor regions is growing rapidly, and many chromosomes appear to have such regions lost at fairly high frequencies in EOC. The 11q23-qter region has received much recent interest as the site of a putative tumor suppressor gene(s) in cancers of the ovary (1, 2), breast (3–5), lung (5), colon (7), cervix (8), and cutaneous malignant melanoma (9, 10). The region was shown to be significantly associated with advanced stage and poor prognosis in cancers of the ovary (2) and breast (11). The recent cloning and localization to chromosome 11q of the ataxia telangiectasia gene (12) (thought to contribute as a modifying gene to perhaps 20% of breast cancer) provides a plausible candidate in this region, which is increasingly described in solid tumors.

The present study refines the LOH map of the 11q22-qter region in EOC using selected tumors known to have loss somewhere on chromosome 11, concentrating on the subregion of LOH previously identified and associated with adverse survival (2).

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[2] Supported by a grant from the Scottish Hospitals Endowments Research Trust.

[3] The abbreviations used are: EOC, epithelial ovarian cancer; LOH, loss of heterozygosity; SRO, shortest region of overlap; FIGO, International Federation of Gynecology and Obstetrics.

## Materials and Methods

**Patient Population and Tumor Samples.** Fresh primary ovarian tumor tissue from 40 patients with ovarian tumors (31 with epithelial ovarian cancer) was obtained as previously described (2). FIGO staging, histopathology, and differentiation state were determined and reviewed in a standardized fashion at a multidisciplinary combined gynecological oncology clinic. Treatment was planned and delivered in accordance with standard protocols, and follow-up data on this group are complete. Minimum and maximum follow-up on living patients are 33 and 52 months, respectively; all deaths that have occurred have been due to ovarian cancer. Patient characteristics are outlined in Table 1.

Primary tumor and constitutive DNA were extracted using standard methods as previously described (13). In the present study, all tumors were known to have LOH somewhere on chromosome 11, including 11p and proximal 11q in cases H59, G56, G43, H5, G47, H55, G42, G46, and G18, prior to refinement of the telomeric region (2).

**LOH Analysis.** DNA samples were analyzed as normal/tumor pairs by PCR using primers for the following CA repeat polymorphic microsatellites lying in the region 11q22 to 11q24.3: *D11S35, D11S925, D11S1336, D11S933, D11S934, D11S707, D11S1351, D11S912, D11S1320,* and *D11S968.* Primer sequences were obtained from the Genome Data Base. The PCR products were resolved by electrophoresis on a 6 or 9% denaturing urea/polyacrylamide gel, passively transferred to Hybond nylon and probed with $\gamma-^{32}P$ end-labeled $(CA)_{35}$ oligonucleotide as previously described (14). LOH was initially scored on the basis of independent visual reporting by two observers who registered for clear reduction in intensity of one tumor allele. Computerized densitometric analysis of the autoradiographs was then performed on a high-resolution flat bed scanner using a Bioimage whole band analyzer software system (v3.2.2) run on a SUN SPARC UNIX platform. The relative ratio of alleles was determined, normalized, and compared. Where the tumor allele ratio differed from the normal allele ratio by 30% or more ($r \leq 0.7$), LOH was assigned, as previously described (4).

**Statistical Analysis.** Two-tailed Fisher's exact test was used to look for associations between LOH regions and clinicopathological parameters. Kaplan-Meier analysis and the log rank test were used to analyze overall

Table 1 *Clinicopathological characteristics of the study cohort*

| | |
|---|---|
| Number of patients | 40 |
| Previously known to have chromosome 11 LOH | 40 |
| Ovarian adenocarcinoma (EOC) | 31 |
| Histology | |
| Serous | 16 |
| Endometrioid | 10 |
| Mucinous | 4 |
| Clear cell | 1 |
| Differentiation | |
| Well | 2 |
| Moderate | 12 |
| Poor | 17 |
| Stage | |
| I/II | 11 |
| III/IV | 19 |
| Not known | 1 |
| Benign adenofibroma | 3 |
| Borderline Malignant potential | 3 |
| Granulosa tumor | 2 |
| Teratoma | 1 |

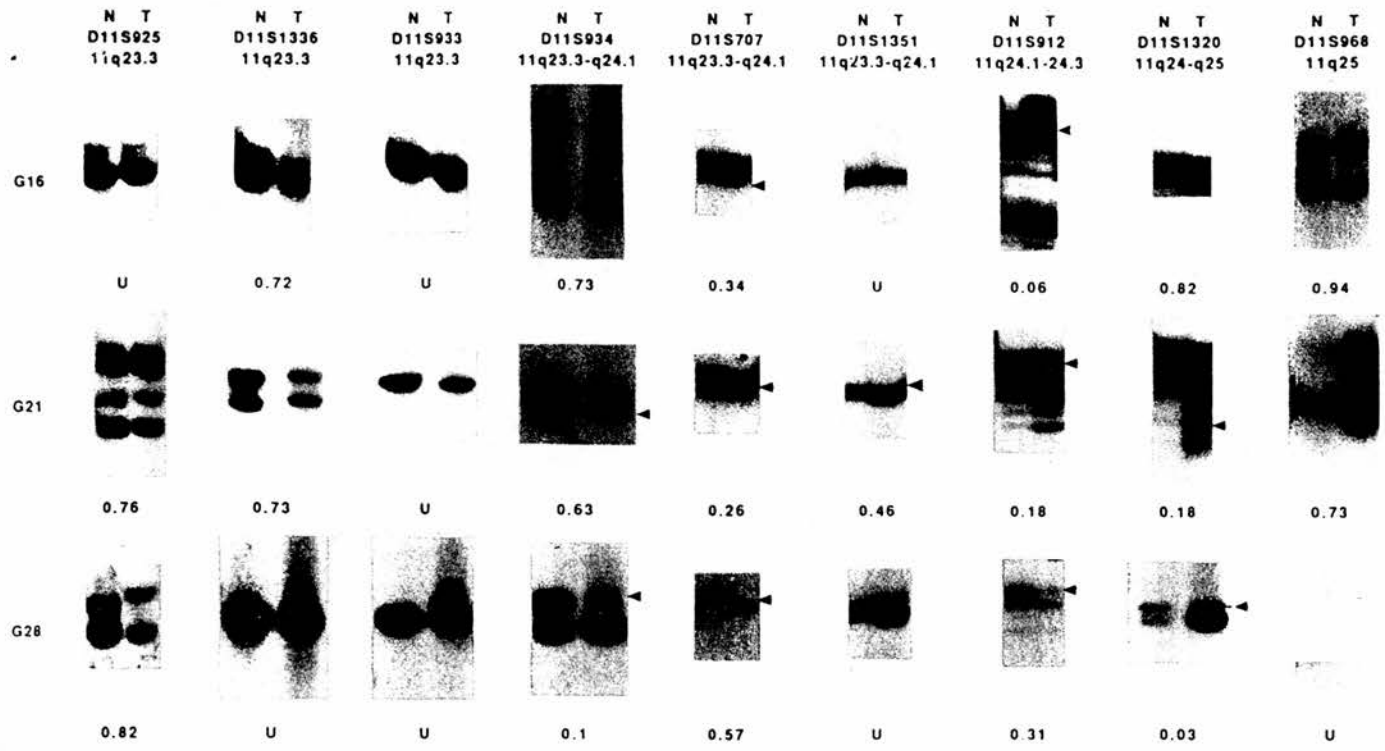


Fig. 2. Primary LOH data from three cases critical to the definition of the 11q23.3–q24 locus. *N*, normal DNA; , tumor DNA shown at *top* from patients G16, G21, and G28 (*left*). Microsatellite loci are shown form centromeric (*top left*) to telomeric (*top right*). *Arrows,* alleles showing LOH (allele imbalance). Densitometric ratios of allele intensity were calculated (*shown at bottom*), and values between 0.0 and 0.7 are taken to indicate LOH. *U*, uninformative (homozygous). Note microsatellite instability at *D11S925* for patient G26.

survival (diagnosis to death) for those patients with *versus* those without LOH in defined regions of chromosome 11q.

## Results

**Molecular Analysis.** Clinical and pathological characteristics of the cohort are outlined in Table 1. LOH was detected somewhere on chromosome 11 in all 40 tumors in this series (selected for detailed analysis from our previous study). H59, G56, G43, H5, G47, H55, G42, G46, and G18 all had been shown to have chromosome 11 LOH outside the 11q22–q25 region from a previous analysis (Ref. 2; data not shown). Data from *D11S35, D11S925,* and *D11S912* have been included from this previous report for most of the tumors, although the latter two microsatellite PCR reactions were repeated for this study.

Fig. 1 shows that two separate regions of LOH are identified within the 11q22–q25: the minimum extent of the telomeric region of LOH (11123.3–24.3) is defined by tumor G16. The centromeric extent of this region is defined by *D11S934.* and the telomeric extent is defined by *D11S1320* (Figs. 1 and 2). The size suggested for this region from a recently published radiation hybrid map is 169 centirays (about 8.5 Mb; Ref. 15). Within this region, the frequency of LOH at *D11S912* was 11 (58%) of 19 EOCs and 0 of 5 benign/borderline; at *D11S707* it was 9 (69%) of 13 in EOCs and 0 of 1 borderline. LOH involving this distal deletion unit occurred in 18 (58%) of 31 EOCs.

The large centromeric region of LOH lies between *D11S35* (11q22) and *D11S925* (11q23.3) as defined by tumors G36, G17, G34, and G55 (Fig. 1). LOH involving this centromeric deletion unit occurred in 13 (42%) of 31 EOCs.

Only a minority of tumors exhibited LOH at either 11q22–q23.3 (4/31, 13%) or 11q23.3–24.3 (8/31, 26%) alone. Nearly 30% of the cases simultaneously exhibited LOH at both 11q22–q23.3 and 11q23.3–q24.3 (fig. 1).

**Statistical Analysis.** Fisher's exact test was used to determine whether loss from these two regions was significantly associated with the clinicopathological features of ovarian cancer. No significant correlations were seen between regional losses and FIGO stage ($P = 0.15$), histological type, or differentiation grade. The centromeric 11q22–q23.3 region showed no statistical association with survival for regional LOH alone or in combination with other regions. However, LOH of the 11q23.3–q24.3 region, either alone or in combination with LOH in other regions, showed a strongly significant correlation with survival by Fisher's exact test ($P = 0.004$; alive *versus* dead patients with 33-month minimum follow-up), despite all of the tumors in the series exhibiting LOH somewhere on chromosome 11, confirming our previous finding for *D11S912* (2).

Kaplan-Meier/log rank analysis showed no survival difference ($P = 0.632$) for the centromeric region of LOH (Figure. 3*A*) but the distal LOH region (11q23.3–q24.3) between *D11S934* and *D11S1320* correlated signifficantly with adverse actuarial survival (log rank test, $p = 0.011$; Figure 3*B*). However, the log rank analysis was not significant when only advanced FIGO stage cancers were considered in relation to 11q23.3–q24.3 LOH status ($p = 0.09$).

**Other Tumor Types.** We have included data from a benign ovarian teratoma, regarded as haploid parthenogenetic, demonstrating LOH at all informative chromosome 11 loci. This tumor also had clear LOH at all informative chromosome 17 loci tested (data not shown) and provides a useful positive LOH control.

Benign and borderline epithelial tumors showed no case with secure LOH involving the *D11S934–D11S1320* distal SRO. The two granulosa cell tumors had minimal LOH located outside the critical region.

## Discussion

This study demonstrates that two regions of LOH within 11q22–q25 can be defined in ovarian tumors. As has been shown from previous studies (1, 5, 16), LOH in this region appears to correlate with genetic loss rather than amplification.

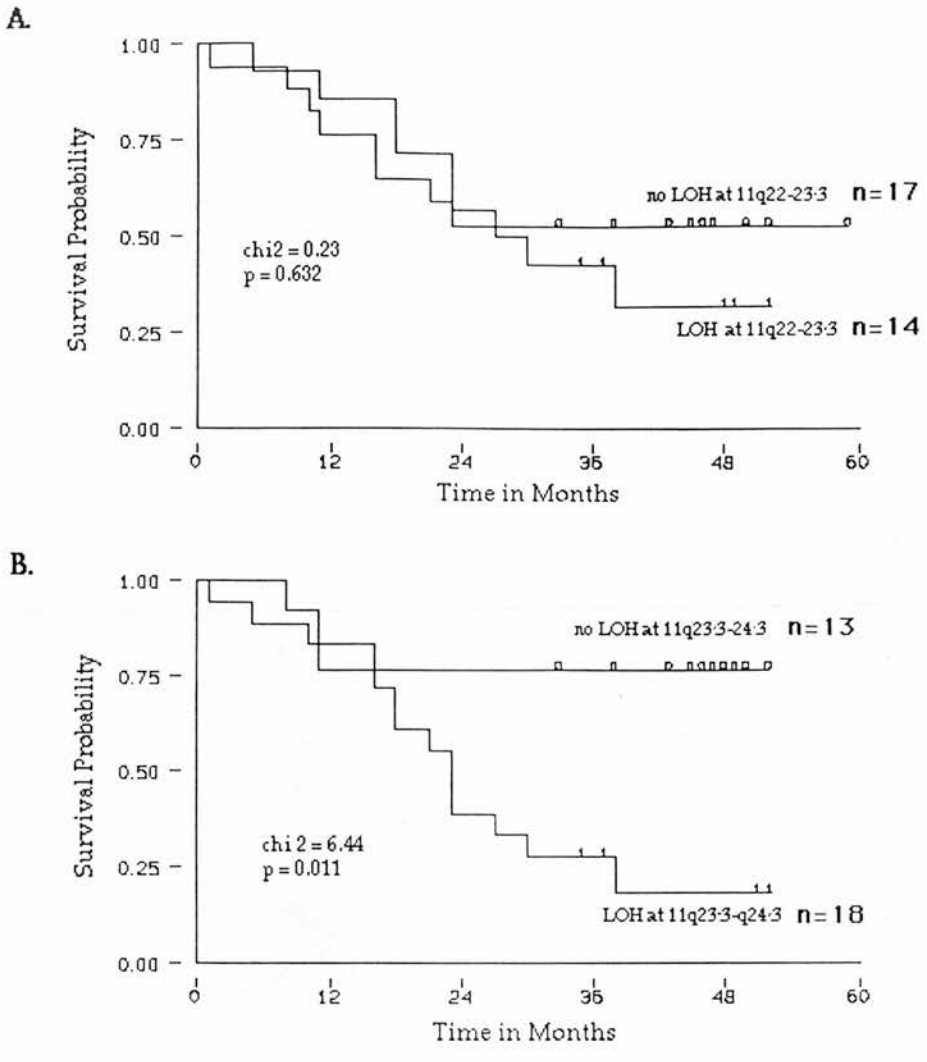


Fig. 3 Kaplan-Meier survival curves comparing survival after a diagnosis of ovarian cancer in those with or without LOH at 11q22–q23.3 (*A*) and in those with or without LOH at 11q23.3–q24.3 (*B*). LOH at 11q23.3–q24.3 shows significantly reduced actuarial survival ($P = 0.011$, log rank test), indicating an aggressive disease course. *Tick marks*, census survival times.

The proximal SRO (which is a large region defined by only two microsatellites in our study) contains the ataxia telangiectasia and progesterone receptor genes and is lost frequently (in 40% of cases), but does not crudely correlate with survival or other adverse prognostic features in EOCs, although it is associated with low tumoral progesterone receptor content (17). This proximal region broadly corresponds to a region that has recently been identified in breast cancer (3–5).

The telomeric 11q23.3–q24.3 region, which we concentrated on due to previous association of *D11S912* LOH with poor prognosis, excludes the *ATM* locus and the region recently mapped in breast cancer. The SRO has been narrowed by our analysis to 8.5 Mb lying between *D11S934* and *D11S912* at 11q23.3–q24.3 based on recently published physical maps (15, 18). Kaplan-Meier survival analysis revealed that LOH involving this 8.5-Mb region is significantly associated with poor survival in EOCs. However, when only the advanced stage tumors were considered with this telomeric locus, the log rank test did not reach significance, although Kaplan-Meier analysis showed a trend toward worse survival in those with LOH of the distal region. This suggests that a late-acting progression-suppressor may be located within this interval. LOH within this region should be subjected to a larger prospective analysis to assess its role as an independent prognostic factor and its value in assessing survival prospectively. The distal deletion interval currently contains five candidate genes: (from centromeric to telomeric) *SRPR, ETS-1, FLI1, ZNF1,* and *NFRKB* (15). The possibility that high-frequency nonspecific genome instability might be responsible for the observed regional losses was controlled by selecting tumors with chromosome 11 loss preferentially, making it unlikely that the observed survival association in this population could be attributed to random high-frequency genetic alteration. Additionally, the retention of heterozygosity telomeric to the 11q23.3–q24.3 LOH region suggests that LOH at this distal unit is not simply due to high-frequency non specific telomeric breaks.

The development of comparative genomic and expression techniques as they impact on positional and functional cloning strategies should allow identification of genes from these critical regions.

## References

1. Foulkes, W. D., Campbell, I. G., Stamp, G., and Trowsdale, J. Loss of heterozygosity and amplification on chromosome-11q in human ovarian-cancer. Br. J. Cancer, *67*: 268–273, 1993.
2. Gabra, H., Taylor, L., Cohen, B. B., Lessels, A., Eccles, D. M., Leonard, R. C. F., Smyth, J. F., and Steel, C. M. Chromosome 11 allele imbalance and clinicopathological correlates in ovarian tumours. Br. J. Cancer, *72*: 367–375, 1995.
3. Carter, S. L., Negrini, M., Baffa, R., Gillum, D. R., Rosenberg, A. L., Schwartz, G. F., and Croce, C. M. Loss of heterozygosity at 11q22–q23 in breast cancer. Cancer Res., *54*: 6270–6274, 1994.
4. Hampton, G. M., Mannermaa, A., Winquist, R., Alavaikko, M., Blanco, G., Taskinen, P. J., Kiviniemi, H., Newsham, I., Cavenee, W. K., and Evans, G. A. Loss of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. Cancer Res., *54*: 4586–4589, 1994.
5. Negrini, M., Rasio, D., Hampton, G. M., Sabbioni, S., Rattan, S., Carter, S. L., Rosenberg, A. L., Schwartz, G. F., Shiloh, Y., Cavenee, W. K., and Croce, C. M.

Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. Cancer Res., *55:* 3003–3007, 1995.

6. Rasio, D., Negrini, M., Maneneti, G., Draggani, T. A., and Croce, C. M. Loss of heterozygosity at chromosome 11q in lung adenorcarcinoma: identification of three independent regions. Cancer Res., *55:* 3988–3991, 1995.
7. Keldysh, P. L., Dragani, T. A., Fleischman, E. W., Konstantinova, L. N., Perevoschikov, A. G., Pierotti, M. A., Della Porta, G., and Kopnin, B. P. 11q deletions in human colorectal carcinoma: cytogenetics and restriction fragment polymorphism analysis. Genes Chromosomes Cancer, *6:* 45–50, 1993.
8. Hampton, G. M., Penny, L. A., Baergen, R. N., Larson, A., Brewer, C., Liao, S., Busby-Earle, R. M. C., Williams, A. W. R., Steel, C. M., Bird, C. C., Stanbridge, E. J., and Evans, G. A. Loss of heterozygosity in cervical carcinoma: subchromosomal localisation of a putative tumor-suppressor gene to chromosome 11q22–q24. Proc. Natl. Acad. Sci. USA, *91:* 6953–6957, 1994.
9. Tomlinson, I. P. M., Gammack, A. J., Stickland, J. E., Mann, G. J., MacKie, R. M., Kefford, R. F., and McGee, J. O. Loss of heterozygosity in malignant-melanoma at loci on chromosome-11 and chromosome-17 implicated in the pathogenesis of other cancers. Genes Chromosomes & Cancer, *7:* 169–172, 1993.
10. Herbst, R. A., Larson, A., Weiss, J., Cavenee, W. K., Hampton, G. M., and Arden, K. C. A defined region of loss of heterozygosity at 11q23 in cutaneous malignant melanoma. Cancer Res., *55:* 2494–2496, 1995.
11. Winqvist, R., Hampton, G. M., Mannermaa, G., Blanco, G., Alavaikko, M., Kiviniemi, H., Taskinen, P. J., Evans, G. A., Wright, F. A., Newsham, I., and Cavenee, W. K. Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res., *55:* 2660–2664, 1995.
12. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanol, O., Lavin, M. S., Jaspers, N. J., Taylor, A. R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science (Washington DC), *268:* 1749–1753, 1995.
13. Eccles, D., Cranston, G., Steel, C. M., Nakamura, Y., and Leonard, R. C. F. Allele losses on chromosome 17 in human epithelial ovarian cancer. Oncogene, *5:* 1599–1601, 1990.
14. Cohen, B. B., Wallace, M. R., and Crichton, D. N. A comparison of procedures for analysing microsatellite repeat polymorphisms. Mol. Cell Probes, *6:* 439–442, 1992.
15. James, M. R., Richard, C. W. III, Schott, J-J., Youstry, C., Clark, K., Bell, J., Terwilliger, J. D., Hazan, J., Dubay, C., Vignal, A., Agrapart, M., Imai, T., Nakamura, Y., Polymeropoulos, M., Weissenbach, J., Cox, D. R., and Lathrop, G. M. A radiation hybrid map of 506 STS markers spanning human chromosome 11. Nat. Genet, *8:* 70–76, 1994.
16. Gudmundsson, J., Barkardottir, R. B., Eiriksdottir, G., Baldursson, T., Arason, A., Egilsson, V., and Ingvarsson, S. Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. Br. J. Cancer, *72:* 696–701, 1995.
17. Gabra, H., Langdon, S. P., Watson, J. E. V., Hawkins, R. A., Cohen, B. B., Taylor, L., Mackay, J., Steel, C. M., Leonard, R. C. F., and Smyth, J. F. Loss of heterozygosity at 11q22 correlates with low progesterone receptor content in epithelial ovarian cancer. Clin. Cancer Res., *1:* 945–953, 1995.
18. Van Heyningen, V., and Little, P. F. R. Report of the fourth international workshop on human chromosome 11 mapping 1994. Cytogenet. Cell Genet., *69:* 128–158, 1995.